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(57) Abstract

The present invention relates to a novel expression system which allows the study of experimental genes of interest on cellular events soon after transfection. The expression system includes a vector which encodes for a recombinant antibody binding unit (rAb). The expression system enables identification and selection of transfected cells from culture to be carried out immediately, within hours, after the transfection event. The invention also relates to cells transfected with the expression system and methods for selection and isolation of cells transfected with the expression system.

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NOVEL SYSTEM FOR ISOLATING AND IDENTIFYING EUKARYOTIC CELLS TRANSFECTED WITH GENES AND VECTORS

BACKGROUND OF THE INVENTION

This invention was made with Government support under Grant No. DK48845 with the National Institutes of Health (NIH). The Government may have certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates generally to the fields of cell biology, molecular biology and immunology and, more specifically, to a novel system of identifying and isolating cells transfected with vectors encoding genes of interest. Use of this novel system allows rapid selection of transfected cells from total populations of cells in culture.

BACKGROUND INFORMATION

Introduction

Recent advances in molecular biology have allowed the production of recombinant immunoglobulin molecules (rAbs) from existing hybridomas, as described in Morrison, S.L., *et al.*, *Clin. Chem.* 34:1668 (1988); Orlandi, R., *et al.*, *Proc. Natl. Acad. Sci.* (1989); Larrick, J.W., *et al.*, *Biochem. Biophys. Res. Commun.*

160:1250 (1989) and *de novo* from phage display libraries as described in McCafferty, J., *et al.*, *Nature* 348:552 (1990); Clackson, T., *et al.*, *Nature* 352:624 (1991); Marks, J.D., *et al.*, *J. Mol. Biol.* 222:581 (1991); Hoogenboom, H.R., *et al.*, *Nucl. Acids Res.* 19:4133 (1991); Winter, G. *et al.*, *Annu. Rev. Immunol.* 12:433 (1994). Recombinant immunoglobulin molecules (rAbs), including single chain antibodies (sFvs) and Fabs, are able to bind their cognate antigens with high specificity and affinity, as described in Winter, G., *et al.*, *Annu. Rev. Immunol.* 12:433 (1994). These modular binding regions can be fused with bioactive proteins or drugs and used to direct these molecules to their intended site of action, as described in Siegall, C.B., *et al.*, *J. Immunol.* 152:2377 (1994). By using phage display technology, rAbs can now be isolated and produced *in vitro* against molecules, both natural and synthetic, that are either non-immunogenic or of such a high toxicity as to preclude their production *in vivo*, as described in McCafferty, J., *et al.*, *Nature* 348:552 (1990); Clackson, T., *et al.*, *Nature* 352:624 (1991); Hoogenboom, H.R., *et al.*, *Nucl. Acid Res.* 19:4133 (1991); Marks *et al.*, *J.D., J. Mol. Biol.* 222:581 (1991); Winter, G., *et al.*, *Annu. Rev. Immunol.* (1994). The power and versatility of these proteins allows rAbs to be used in ways that conventional antibodies could not.

The present invention uses such recombinant antibody binding units, in conjunction with expression vectors coding for genes of interest, as "molecular hooks" to identify and separate transfected cells from a

culture. The present invention allows for identification and selection of transfected cells as early as two hours after transfection, thus allowing study of the acute effects of the expression of the gene of interest.

The use of the invention's "molecular hooks" will assist in the identification and characterization of many cellular signaling factors heretofore not possible with current technology. Such identification and characterization has been possible only as a result of the development of technology enabling the introduction of expression plasmids into mammalian cells. The subsequent examination of the effect (on cellular growth and differentiation) of constitutively expressing an otherwise tightly regulated molecule has permitted the elucidation of many complex signaling pathways. With current technology, not all of the functional characteristics of signaling molecules are readily detectable using these systems. For example, it would be of great value to study the effect of dominant negative mutations of signaling molecules in both transformed and primary cells. Those negative or toxic mutations that result in inhibition of cell growth or cell death may be masked due to the low efficiency of transfection. In addition, it is not possible to increase the population of cells expressing a gene of interest by selecting for stable transformants as negative growth phenotypes are not amenable to this type of selection. This limitation of current technology in expression systems has, to a limited extent, been addressed by the use of inducible promoter systems, see, for example, those described in

Levinson, A.D., "Gene Expression Technology," *In* D.V. Goeddel (Ed.), *Methods in Enzymology*, Academic Press, p. 497 (1991). However, this approach is not always optimal or applicable and has met with varied success depending on the cell type and origin of the promoter utilized. If cells expressing dominant-negative signaling molecules could be selected from culture soon after, within hours, of transfection, rather than days or weeks later, as is the case with current technology, assessment of the effects of the expression of a potentially negative effector would be possible. Similarly, early enrichment of transfected cells would allow studies of acute expression of transfected genes in homogeneously expressing cell cultures.

Selection of primary cell cultures that do not divide, such as neuronal cell cultures, have been limited to techniques that involve negative selection, such as antibiotic resistance conferred by the transfected vector. Selection of transfected cells by utilizing resistance to antibiotics takes days. In contrast, selection of primary cultures with the vectors of the instant invention allows selection as soon as 2 hours after the transfection event, depending on the primary cell culture.

The present invention is a novel alternative technology, encompassing a new expression system that will enable selection of transfected cells from culture to be carried out soon after, within 2 hours, of the

transfection event, along with other advantages that will become apparent below.

The present invention satisfies these needs and provides related advantages as well.

SUMMARY OF THE INVENTION

The present invention relates to a eukaryotic expression vector for the identification and separation of transfected cells from a total cell population, comprising: a first DNA sequence encoding an anti-hapten recombinant antibody, said recombinant antibody capable of binding a specific hapten; a second DNA sequence encoding for a transmembrane domain functionally linked to said first DNA sequence; a third DNA sequence encoding for a signal sequence functionally linked to said first DNA sequence; a first promoter operatively linked to said first DNA sequence; a fourth DNA sequence encoding for at least one protein; a promoter operatively linked to said fourth DNA sequence.

The invention also relates to a mixture of eukaryotic expression vectors for the identification and separation of transfected cells from a total cell population comprising a first vector which in turn comprises: a first DNA sequence encoding an anti-hapten recombinant antibody, said recombinant antibody capable of binding a specific hapten; a second DNA sequence encoding for a transmembrane domain functionally linked to said first coding sequence; a third DNA sequence

encoding for a signal sequence functionally linked to said first DNA sequence; and a promoter operatively linked to said first DNA sequence.

The invention also relates to a method of identifying and isolating transfected cells from the total cell population, comprising: transfecting a eukaryotic cell with a eukaryotic expression vector; exposing said cell to a hapten conjugated to a cell selection means; separating said cell, bound to said selection means, from the total cell population.

The invention also relates to a kit for the identification and separation of transfected cells from a total cell population, comprising a eukaryotic expression vector and a cell separation means.

The invention also relates to cells transfected with the expression vectors of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B demonstrate features and the plasmid map of the eukaryotic expression vector pPhOx.TM, which encodes for an anti-hapten (anti-phOx) sFv.

Figure 2 demonstrates the *in vitro* transcription and translation product of pPhOx.TM using an SDS polyacrylamide gel autoradiogram. As seen in lane 3, the transcription/translation reaction produced a protein of the expected molecular weight, which is approximately

30kD (phOx sFv) plus 7.6 kD (the PDGFR transmembrane domain), totaling approximately 40kD. Note lane 1 contains the positive control beta-galactosidase encoding DNA and lane 2 contained no exogenous DNA.

Figure 3A demonstrates microscopic inspection of adenovirus-transformed human kidney cells, ATCC # CRL-1573 (designated "293") transfected with pPhOx.TM. 24 hours after transfection, the cells were incubated with phOx-BSA magnetic beads for 30 at 37°C with gentle agitation. Cell binding to antigen (phOx-BSA) coated magnetic beads at 24 hours post-transfection is observed in this micrograph.

Figure 3B demonstrates transfected "293" (ATCC # CRL-1573) and HeLa cells (ATCC # CCL-2) transfected with pPhOx.TM by electroporation. "293" cells can be selected from culture as early as two hours post-transfection with pPhOx.TM, indicating that sFv is displayed on the cell surface at two hours post-transfection. HeLa cell display of pPhOx sFv did not occur until eight hours post-electroporation (transfection).

Figure 3C demonstrates that outer cell membrane expression of sFv can occur in differing cell types. Four cell lines derived from breast tumors and one cell line derived from a malignant melanoma were electroporated with pPhOx.TM and selected with pPhOx-BSA beads at 24 hours. The four breast tumor cell lines, as indicated in Table I, are: (1) MDA-MB-468 (ATCC # HTB-132), a human adenocarcinoma of the breast isolated from

pleural effusion, which expresses EGFR; (2) MDA-MB-453 (ATCC # HTB-131), a human adenocarcinoma of the breast isolated from breast effusion, which expresses HER2/*neu* (3) MCF-7 (ATCC # HTB-22), a human adenocarcinoma of the breast isolated from pleural effusion, which expresses neither EGFR nor HER2/*neu;* and,(4) SKBR-3 (ATCC # HTB-30), a human adenocarcinoma of the breast isolated from malignant pleural effusion, which expresses both EGFR and HER2/*neu*. Selected cells were counted and are presented in comparison with the number of cells surviving the electroporation and with the size of the original population ($2x10^6$ cells). Note that selection efficiency varied from cell line to cell line. Increased selection efficiency can be obtained by optimizing transfection conditions for each cell line.

Figure 4 demonstrates that virtually all of the cells that express the sFv fusion protein are efficiently selected from culture using the pPhOx-BSA coated magnetic bead cell selection means. SKBR-3 and MDA-MB-453 cells were transfected and selected with phOx/BSA coated magnetic beads at 24 hours post-transfection. Cellular proteins were then separated by size using an SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred by immunoblot to a nitrocellulose membrane and reacted with radiolabeled antibodies able to bind sFv. Note in the "unselected" lane, meaning cells that did not bind to the magnetic beads, virtually no sFv is detected, indicating that all cells that were transfected were separable from the total

cell culture using the cell separation means (the coated magnetic beads).

Figure 5 demonstrates the efficiency of coexpression of pPhOx.TM and beta-galactosidase. SKBR-3 cells were co-transfected with pPhOx.TM and a vector expressing the gene for β-galactosidase, named pCMVβ, (Clontech, Palo Alto, CA). One third of each transfection reaction was plated in each chamber of a four chamber microscope slide (Nunc, Napierville, IL). Details of the experiment are described in Example III(e) below. Panel A shows mock transfected cells; panel B shows cells transfected with pPhOx.TM alone; panel C shows cells transfected with pCMVβ (β-galactosidase expressing; and panel D shows cells transfected with both pPhOx.TM and pCMVβ.

The results demonstrate that most if not all of the cells expressing the functional pPhOx.TM product (cells with silver grains, denoted by arrows) are also expressing β-galactosidase (blue staining, the point of the triangles opposite the stars points towards representative cells staining for β-galactosidase). Greater than 98% of the cells selected with pPhOx-BSA-coated magnetic beads also stained positively for protein product of the experimental gene of interest, in this experiment, the β-galactosidase gene.

Figure 6 sets forth the DNA sequence of pPhOx.TM.

Figur 7 sets forth the DNA sequence of pCR™3*lacZ*.

DETAILED DESCRIPTION OF THE INVENTION

In the following description, reference will be made to various methodologies known to those skilled in the art of molecular genetics, immunology and general biology. Publications and other materials, as cited herein, setting forth such known methodologies to which reference is made, are incorporated herein by reference in their entireties as though set forth in full.

General principles of antibody engineering are set forth in *Antibody Engineering*, 2nd edition, Ed. C.A.K. Borrebaeck, Oxford Univ. Press (1995). General principles of protein engineering are set forth in *Protein Engineering*, A Practical Approach, Ed. Rickwood, D., *et al.*, IRL Press at Oxford Univ. Press, Oxford, Eng. (1995). General principles of antibodies and antibody binding to haptens are set forth in: Nisonoff, A., *Molecular Immunology*, 2nd edition, Sinauer Associates, Sunderland, MA (1984); and, Steward, M.W., *Antibodies, Their Structure and Function*, Chapman and Hall, New York, NY (1984).

The present invention generally relates to a novel system of identifying and separating cells transfected with a gene of interest. Such a system allows the study of experimental genes of interest on cellular events soon after transfection, as described above in the Summary. In a preferred embodiment, cells transfected with the expression system of the invention can be selected and experimented on as soon as 2 hours post-transfection.

This new technology, the present invention, thereby aids in the identification and characterization of genes of experimental interest soon after transfection. Intracellular signaling proteins and dominant-negative signaling molecules are now accessible to study. Early events initiated by dominantly acting oncogenes, negatively acting tumor suppressors, as well as temporal events along differentiated pathways can now be studied.

For example, signaling pathways in cell lines derived from a certain tumor type can be studied with the present invention. The invention can be used to study the role of the HER-2/neu oncogene in breast carcinoma by expressing dominant negative mutations of signaling proteins in breast cancer cell lines. HER-2/neu (c-erbB-2) is overexpressed in 30% of breast tumors and its presence is correlated with lower survival rates of patients with these tumors (Elledge, R.M., *et al.*, *Seminars in Oncology* 19:244 (1992). The HER-2/neu protein demonstrates close sequence homology with, but is distinct from, the epidermal growth factor receptor (EGFR) (Scheuter, A.L., *et al.*, *Science* 229:976 (1985). The unregulated growth characteristics of HER-2/neu-positive tumors is hypothesized to arise, at least in part, from the effect of HER-2/neu on intracellular signaling pathways (Kumar, R., *et al.*, *Mol. Cell. Biol.* 11:979 (1991)). The invention described herein can be used to isolate homogeneous populations of cells expressing dominant negative mutations of cellular signaling proteins known to interact with the EGF receptor such as PI3K, PLCγ1, Grb2, Syp, Nck, Shc, and

p91 in several cell lines derived from breast tumors (see Table I).

Table 1

Properties of cell lines derived from carcinoma of the breast

Cell Type	EGFR	HER2/*neu*	Tumorigenic in Nude Mice	Derived From
MDA-MB-468	+	--	+	Human adenocarcinoma of breast, from pleural effusion
MDA-MB-453	--	+	--	Human carcinoma of breast from effusion
MCF-7	--	--	+	Human adenocarcinoma of breast, from pleural effusion
SKBR-3	+	+	+	Human adenocarcinoma of breast, from malignant pleural effusion

For another example, efficient study of regulatory proteins, such as early events in the *Ras*-regulated serine/threonine kinase pathways, requires a transfection system that allows rapid selection of transfected cells. The present invention will allow an analysis of when this pathway diverges into the Ras-MEK-MAPK axis and the Ras-MEKK-SEK-SAPK (JNK) axis (Sanchez, I., *et al.*, *Nature* 372:794 (1994); Yan, M., *et al.*, *Nature* 372:798 (1994); Derijard, B., *et al.*, *Science* 267:682 (1995)).

This expression system of the invention, by giving researchers the ability to select cells expressing genes of interest from culture as soon as 2 hours after transfection, allows the study of the acute effects of expression of a wide variety of experimental systems otherwise not accessible to study. For example, dominant negative or constitutively active mutations of proteins involved in signal transduction can be studied using the present invention. Analyses of early transcription events are now accessible to study. Experimentation on the acute effects of transfection on primary cell cultures, including cells that normally do not divide, such as neurons, is now possible.

The present invention relates to a novel system for rapidly isolating and identifying eukaryotic cells after transfection. The invention employs a vector encoding for a "molecular hook," including an rAb or a receptor-like molecule, that is expressed on the cell's surface. Such expression may occur as early as 2 hours after transfection. The rAb binds to a specific "hapten," which, as defined below, can be any unique, selective epitope. Structurally, the rAb can be in the form of double or single chain antibody (sFv), an Fab fragment, or any functional binding unit.

The invention's use of the rAb binding domain on the transfected cell and the hapten on the cell selection means has advantages over the converse option (the hapten expressed on the transfected cell). First, it is advantageous to have a high density of hapten or epitope

on the cell selection means, such as a bead. Second, it is advantageous to have the entity that has a higher level specific binding, i.e. less cross-reactivity with irrelevant molecules, on the cell selection means. The rAb or receptor-like molecule has a greater possibility of cross-reactivity than the hapten or epitope molecule. The cell selection means, with a high hapten density and binding specificity, will yield a relatively pure population of cells transfected with and expressing the requisite rAb or receptor-like molecule.

In another embodiment of the invention, in place of the rAb, the "selective hook" expressed on the cell's surface is a receptor-like or adhesion molecule capable of selectively binding to a specific hapten, epitope or ligand. One skilled in the art would have the means to select receptor-like or adhesion molecule binding domains for purposes of incorporation into the eukaryotic expression vector of the invention. As used herein, the term "receptor-like" molecule means any protein capable of specifically binding a hapten, epitope, or ligand. Examples of protein binding sites, to be expressed on the cell's surface, that can be used to selectively bind epitopes or haptens, include adhesion molecules such as cadherins, selectins, fasciclins, integrins, leukocyte adhesion receptor, neuroglian, VLA family molecules and the like. Examples of protein binding sites that can be used to selectively bind include growth factor receptor binding sites, including growth hormone receptor, insulin receptor, interleukin receptors and the like. Examples of specific protein binding interactions useful in the

instant invention are described in Creighton, T.E., in *Proteins, Structure and Molecular Principles*, W.H. Freeman and Company, New York, NY (1984); and, adhesion molecules are described in Pigott, R., et al., in *The Adhesion Molecule*, Academic Press, Harcourt Brace & Co., New York, NY (1993). These references, as all references cited herein, are incorporated by reference in their entirety.

The rAb and receptor-like or adhesion molecule are also engineered to include coding sequences for a transmembrane domain or any membrane anchoring sequence and a secretion signal (leader sequence), thus allowing its expression on the transfected cell's outer membrane surface (i.e., extracellular expression). All coding sequences include 3' eukaryotic polyadenylation (poly-A) sequences, for the necessary 3' poly-adenylic acid RNA sequence needed.

Once expressed on the cell's outer membrane surface, the rAb or receptor-like domain is capable of binding to a specific hapten or epitope. This hapten or epitope is bound either directly or indirectly to a cell separation means, such as magnetic beads or sheets, tubes, porous matrices, or any natural or synthetic material including metals, polymers, latex beads, agarose, Sepharose, or any solid surface. The hapten or epitope can also include or be conjugated to a fluorescent or other labeled, selectable hapten or epitope. An example is PhOx-BSA-FITC. This allows for identification and selection of the transfected cell

shortly after transfection, which can be as soon as approximately 2 hours after transfection, depending on the experimental system.

The transfected cells can be separated from unbound, untransfected cells by any physical means, such as filtration, isolation, by magnetic field, centrifugation, washing and the like. This rapid enrichment of transfected cells allows studies of the acute expression of the transfected experimental genes of interest.

The eukaryotic expression vector of the invention can use any vector or mixture of vectors capable of transfection and expression of DNA in eukaryotic cells. Such vectors are well known in the art and include, but are not limited to plasmids, viruses (such as adenoviruses, bovine papillomavirus, Epstein Barr virus, papovavirus, and retroviruses) or linear, double-stranded DNA. For example, retrovirus vectors are described in Somia, N.V., *et al.*, *Proc. Natl. Acad. Sci.* 92:7570 (1995). Additional vectors are described in *Catalogue of Recombinant DNA Materials*, 2nd Edition, ATCC, Parklawn, MD (1991); and viral vectors are described in Levinson, A.D., "Expression of Heterologous Genes in Mammalian Cells", In *Methods in Enzymology* 185:485 (1990). One skilled in the art would know how to choose a vector of choice for a particular eukaryotic cell line or experimental system. Vectors are available to one skilled in the art that, upon transfection, are transient and episomal, stable and episomal, or stable and

integrated. The vector containing the experimental gene(s) of interest can be encoded within the same vector as the rAb or can be on another or mixture of other vectors. If a mixture of vectors are used, they are co-transfected.

The rAb is designed to bind to a specific hapten or epitope. As used herein, the term "hapten" or "epitope" means any organic or inorganic molecule capable of being bound by any rAb or recombinant receptor-like molecule, and includes molecule that can serve as a ligand for receptor-like or adhesion molecules. As noted above, by using phage display technology, rAbs can now be isolated and produced *in vitro* against "hapten" molecules, both natural and synthetic, that are either *non-immunogenic* or of such a high toxicity as to preclude their production *in vivo*. If small rigid haptens are used, antibody/hapten affinities as high as 10^{12} M-1 can be generated, as described in Searle, S.J., et al., Antibody Structure and Function, *In* Antibody Engineering, 2nd Ed, Ed. C.A.K. Borrebaeck, Oxford Univ. Press (1995). Thus, for the purpose of this invention, a hapten is defined as not only any molecule that is immunogenic either alone or conjugated to a carrier but any molecule capable of binding to an rAb as described above. Such hapten molecules include aniline derivatives such as: diazonium salts; benzene and derivatives such as dinitrobenzenesulfonate or dinitrobenzene or p-aminobenzenearsonate; phenol and derivatives as dinitrophenol (DNP), DNP-lysine; benzoates and benzoate derivatives such as phenylazobenzoate; acetates and derivatives such

as phenylacetate; and the like. Analysis of haptens and Ab-hapten interactions are described in Nisonoff, A., *Molecular Immunology*, 2nd edition, Sinauer Associates, Sunderland, MA (1984); and, Steward, M.W., *Antibodies, Their Structure and Function*, Chapman and Hall, New York, NY (1984).

As used herein, the term "antibody binding unit" means any functional protein unit which can bind a hapten. Therefore, structurally, the recombinant rAb protein can be designed to take the final form of a double or single chain antibody (designated "sFv"), Fab, Fab' or $F(ab')_2$ fragments, or any functional antigen-antibody binding unit. rAbs, including single chain antibodies (sFvs) and Fabs, are able to bind their cognate antigens with high specificity and affinity, as described in Winter, G., *et al., Annu. Rev. Immunol.* 12:433 (1994). By using phage display technology, rAbs can now be isolated and produced *in vitro* against molecules, both natural and synthetic, that are either non-immunogenic or of such a high toxicity as to preclude their production *in vivo*, as described in: Clackson, T., *et al., Nature* 352:624 (1991); Figini, M., et al., *J. Mol. Biol.* 239:68 (1994); Hawkins, R.E., et al., *J. Mol Biol.* 226:889 (1992); Hoogenboom, H.R., *et al., Immunol. Rev.* 130:41 (1992); Hoogenboom, H.R., *et al., Nucl. Acid Res.* 19:4133 (1991); Jespers, L.S., *et al., Biotechnology* 12:899 (1994); Marks *et al., J.D., J. Mol. Biol.* 222:581 (1991); McCafferty, J., *et al., Nature* 348:552 (1990); Winter, G., *et al., Annu. Rev. Immunol.* 12:433 (1994). The synthesis of single-stranded sFv antibody fragement

gene repetoires is also described by Marks, J.D., "Human Monoclonal Antibodies from V-Gene Repertoires Expressed on Bacteriophage," In *Antibody Engineering*, 2nd Ed, Ed. C.A.K. Borrebaeck, Oxford Univ. Press (1995). Hilyard, K.L. discusses "Protein Engineering of Antibody Combining Sites" In *Protein Engineering*, edited by Rees, A.R. et al., IRL Press at Oxford Univ. Press, New York, NY (1992). As noted above, all references cited herein are incorporated by reference in their entirety.

In the rAb-containing vectors of the invention, the coding sequence for the rAb is operably linked to a strong constitutive promoter capable of expression immediately upon transfection or soon thereafter. As disclosed herein, this enables selection of cells expressing genes of interest, through the extracellular expression of the rAb, within hours after transfection. Such constitutive promoters are well known in the art and include, but are not limited to viral, bacterial or eukaryotic promoters. One skilled in the art would know how to choose a vector of choice for a particular experimental system. Examples of strong constitutive promoters include cytomegalovirus (CMV) immediate early promoter, Rous sarcoma virus (RSV) promoter, adenovirus major late promoter, the lac-inducible promoter, SV40 early promoter and retroviral long terminal repeats (LTRs).

Alternatively, the rAb can be operatively linked to an inducible promoter, such as interferon beta promoter, heat-shock promoter, glucocorticoid promoter

and the like, as generally described in Lewin, B., *Genes V*, Oxford Univ. Press, New York, NY (1994). In this situation, the rAb is expressed on the cell surface and the transfected cell can be identified and isolated from the total cell population as soon as two hours after induction of the promoter.

One skilled in the art would know how to choose additional genetic elements necessary for an experimental system, such as the need to include enhancers within an expression vector, as discussed by Kriegler, M., "Assembly of Enhancers, Promoters, and Splice Signals to Control Expression of Transferred Genes," In *Methods in Enzymology* 185:512 (1990).

One or more genes of interest to be expressed in the transfected cell of the instant invention can be contained within a second vector. The second vector can be co-transfected with the rAb encoding vector. Alternatively, it can be spliced within the rAb-encoding vector.

The experimental gene(s) can be operatively linked to the same or a similar type of strong constitutive promoter as the rAb. Alternatively, it can be operatively linked to a different promoter. This promoter can be an inducible promoter, such as interferon beta promoter, heat-shock promoter, glucocorticoid promoter and the like, as described in Lewin, B., *Genes V*, Oxford Univ. Press, New York, NY (1994). If the gene of interest or the rAb is operatively linked to an

inducible promoter, that rAb or gene can be expressed on the cell's surface as soon as two hours after induction. Alternatively, the experimental gene(s) of interest can be operatively linked to the same promoter as the rAb. This can be effected by inserting an Internal Ribosome Entry Site (IRES) between the coding region for the rAb and the second, downstream, gene (Glass, M. J., *et al.*, *Virology* 193(2):842-852 (1993)).

In designing and synthesizing the promoters, they can be initially placed within the expression vector or genome or can be synthesized in conjunction with the rAb or gene of interest before splicing into their respective vector(s). A polylinker can be designed between the promoter and a poly A sequence for simplified insertion of rAb or gene of interest coding sequences in the expression vector or genome.

In one embodiment of the present invention, the vector of the expression vector is pCR3.1 (Invitrogen, San Diego, CA). pCR3.1 is a eukaryotic expression vector which includes polylinker sites, cytomegalovirus (CMV) promoter, bovine growth hormone (bGH) poly A signal and the ampicillin and neomycin resistance genes for selection, as described in Figure 1.

The rAb sequence is linked to a signal, or leader, sequence that is functional in the transfected host cell. Such signal sequences, also called leader sequences, are well known in the art. A signal sequence is composed of 15-30 amino acids that are relatively hydrophobic, thus

allowing insertion into microsomal membrane. One skilled in the art would know how to choose an appropriate signal (leader) sequence for a particular eukaryotic cell line or experimental system. For example, the leader sequence can be either homologous or heterologous to the transfected host. The desired rAb coding sequence can be linked to any signal (leader) sequence which will allow insertion of the rAb protein in the membrane of the selected host and its expression as a functional, hapten-binding extracellular protein. In one embodiment of the invention, the rAb sFv coding sequence was combined with the murine kappa chain V-J2-C region signal peptide. This signal peptide is described in Coloma, M.J., *et al.*, *J. Immunol. Methods* 152:89 (1992) and Kabat, E.A., *et al.*, *Sequences of Proteins of Immunological Interest*, 4th ed. U.S. Dept. of Health and Human Services. Washington, D.C. (1987).

The rAb and receptor-like coding sequences are also linked to a transmembrane domain, or any membrane anchoring sequence. One skilled in the art would know how to choose an appropriate transmembrane domain sequence for a particular eukaryotic cell line or experimental system. The desired rAb coding sequence can be linked to any transmembrane domain which will allow insertion of the rAb protein in the membrane of the selected host and its expression as a functional, hapten-binding extracellular protein. In one embodiment of the present invention, the rAb coding sequence is combined with the transmembrane domain of the human platelet derived growth factor receptor (PDGFR). The PDGFR

transmembrane domain is described in Gronwald, G.M., *et al., Proc. Natl. Acad. Sci. U.S.A.* 85:3435 (1988).

In one embodiment of the present invention, the expression vector employs a single chain antibody (sFv) directed against a hapten, 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (phOx), to isolate transiently transfected cells from total populations in culture. The fusion protein, phOx sFv, as described in Hoogenboom, H.R., *et al., Nucl. Acids Res.* 19:4133 (1991), also contained two epitope tag peptides (for protein identification by anti-tag antibodies), and the transmembrane domain of the human PDGFR. When expressed in transfected cells, this fusion protein is anchored to the membrane via the transmembrane domain of the PDGFR. The functional antibody binding unit, phOx sFv, is therefore exposed to the extracellular environment. Cells were transiently transfected with an expression vector encoding phOx sFv, designated pPhOx.TM. The cells were then selected from culture using antigen (phOx)-coated magnetic beads (the method for cell separation by magnetic bead is described in detail, see Example III(b) below). Furthermore, when cells were co-transfected with pPhOx.TM and a plasmid containing the gene for β-galactosidase (pCMVβ, Clontech), greater than 98% of the cells selected from culture using the instant method were found to express β-galactosidase activity.

In this embodiment, use of a single-chained rAb, versus a dimeric rAb, is advantageous because the smaller size of the single chain coding sequence allows other

inserted coding sequences to be longer without losing cloning efficiency. Cloning efficiency is inversely α to vector size. For example, if the gene of interest is cloned into the same vector as the rAb, then use of the smaller single-chained rAb allows for the inclusion (insertion) of a longer genes or multiple genes, of interest without increasing the overall size of the vector.

The cell selection means of the instant invention comprises any molecule or device that can be coupled to the hapten of choice and can be used to physically separate transfected cells from culture. For example, the hapten may be coupled directly or indirectly to any insoluble separation agent, including but not limited to magnetic beads, gelatin, glass, Sepharose macrobeads or dextran microcarriers such as Cytodex® (Pharmacia, Uppsala, Sweden). The hapten may be coupled, either directly or indirectly, to plates, tubes, bottles, flasks, magnetic beads or sheets, tubes, porous matrices, or any natural or synthetic material including metals, polymers, latex beads, agarose, Sepharose, or any solid surface and the like. Any molecule or reagent may be used to link to hapten of choice to the cell separation means, including lectins, avidin/biotin, inorganic or organic linking molecules and the like. The cell separation means may utilize antibodies specific for any chemical or biological reagent and any form of detection system known in the art. For example, methods of manufacturing antibodies and utilizing antibodies in detection and separation systems are described in

Antibodies, A Laboratory Manual, edited by E. Harlow *et al.*, Cold Spring Harbor Labs, Cold Spring Harbor, New York (1989), which incorporated by reference in its entirety. The transfected cells can be separated from unbound, untransfected cells by any physical means, such as filtration, isolation, by magnetic field, centrifugation, washing and the like.

The transfection of any expression system can be effected by any means, physical or biological. Physical means include direct injection, or, DEAE-dextran mediated transfection, electroporation, calcium phosphate mediated or lipid-mediated transfection and the like.

The invention also relates to cells transfected with the expression vector and methods for selection and isolation of cells transfected with the expression system.

The following examples are intended to illustrate, but not limit, the present invention.

EXAMPLE I

Cloning Strategy for the Generation of Vector Capable of Expressing Single Chain Antibody Directed Against Hapten

This example describes methods for the generation of a vector capable of expressing a single chain antibody directed against a hapten.

a. Construction of pPhOx.TM

The parent vector for pPhOx.TM is pCR3.1 (Invitrogen, San Diego, CA), a eukaryotic expression vector containing the cytomegalovirus (CMV) promoter, bovine growth hormone (bGH), poly A signal and the ampicillin and neomycin resistance genes for selection, as described in Figure 1A.

A DNA fragment encompassing the nucleotides encoding amino acids 514-562 of the human platelet-derived growth factor receptor (PDGFR) was amplified using nucleotide primers. PDGFR is described in Gronwald *et al., Proc. Natl. Acad. Sci. U.S.A.* 85:3435 (1988). These primers incorporate restriction sites and the Myc.1 epitope tag EQKLISEEDLN, recognized by the monoclonal antibody 9E10.2, as described in Evan, G.I., *et al., Mol. Cell Biol.* 5:3610 (1985). This fragment was cloned into the T/A cloning vector pCRII (Invitrogen, San Diego, CA) and sequenced entirely on both strands to verify integrity. The PDGFR transmembrane fragment was constructed to contain a unique Sal I restriction site at the 5' end that is in the same reading frame as a Sal I site introduced at the 3' end of the phOx sFv sequence. This fragment was also constructed to contain a Not I site at its 3' end immediately following a stop codon which follows amino acid 562 of the human PDGFR sequence. The PDGFR DNA fragment was excised from the pCRII vector by digestion with Sal I and Not I, purified by standard procedures, and ligated into Sal I/Not I digested pCR3.1 vector thereby creating the vector pCR3.1.1.

The sequence encoding the murine Ig kappa-chain V-J2-C-region signal peptide (METDTLLLWVLLLWVPGSTGD) containing an EcoRV site at its 5' end, an influenza hemagglutinin (HA) epitope tag (YPYDVPDYA), and Sfi I and Sal I sites at its 3' end was then subcloned from another sFv-containing vector (pCR3.2) as an EcoRV to Sal I fragment (sFv is a single-stranded antibody specific for 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one, also designated phOx). This fragment was then ligated with EcoRV/Sal I digested pCR3.1.1 creating the vector pCR3.1.2.

The anti-phOx sFv was amplified from the phage display vector pHEN-I (phOx) (Hoogenboom et al., 1991) using primers that encompassed the Sfi I site on the 5' end of the sFv and incorporated a Sal I site on the 3' end of the 3' Myc.1 tag already present in pHEN-I. The PCR product was cloned into pCRII and its sequence integrity determined by dideoxy sequencing. The resulting clone was then digested with Sfi I and Sal I, purified by standard procedures, and ligated with Sfi I/Sal I digested pCR3.1.2 creating pPhOx.TM, as illustrated in Figures 1A and 1B. As a result of the cloning strategy, the Myc.1 epitope tag was fused to the carboxyl-terminal end of the anti-phOx sFv as a tandem repeat. The HA epitope tag (recognized by the monoclonal antibody 12CA5, Boehringer Mannheim, Indianapolis, IN) was fused to the amino terminus immediately after the leader peptide cleavage site such that it is the first sequence in the mature protein. The two epitope tag peptides, one 3' and one 5' to the sFv, were included as

controls for complete expression and membrane display of the fusion protein. Expression of the sFv/PDGFR fusion protein from this plasmid is driven by the cytomegalovirus (CMV) promoter, the sequence of which is included in Figure 6.

b. *In vitro* transcription/translation of pPhOx.TM

As an assay for the integrity of the sFv:PDGFR sequence, the fusion protein was expressed from pPhOx.TM *in vitro* using a rabbit reticulocyte lysate system (Novagen, Inc., Madison, WI), as illustrated in Figure 2. Production of an RNA transcript in this system relied on the T7 promoter that is found between the CMV promoter and the sFv sequence in pPhOx.TM. The protein translated from the resulting message is approximately 40 kD. The expected molecular weight of the phOx sFv:PDGFRTM fusion protein is approximately 37.6 kD (30 kD (phOx sFv) + 7.6 kD (PDGFR TM domain, amino acids 514-562)).

EXAMPLE II

Synthesis of a Hapten Capturing Agent

This example describes methods for the synthesis of a hapten capturing agent through its coupling to a cell separation means.

a. Coupling of the hapten phOx to BSA

4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (phOx) (Sigma, St. Louis, MO) was coupled to bovine serum albumin (BSA) as described previously by Makela *et al.*, *J. Exp. Med.* 148:1644 (1978). By analysis of the UV absorbance spectra of the product and comparison with the molar extinction coefficient (ϵ) of PhOx (where concentration = absorbance at 352 nm / ϵ), it was determined that under these conditions a coupling efficiency of 20 moles of phOx per mole of BSA was achieved.

b. Coupling of phOx-BSA a cell separation means, tosyl-activated magnetic beads

The phOx-BSA conjugate described above was coupled to tosyl-activated magnetic beads (Dynabeads M-450, Dynal, Inc.) using the manufacturer's recommended protocol. Beads were suspended in 50 mM $NaHCO_3$, pH 9.5 to a concentration of $2x10^8$ beads/ml. PhOx-BSA was added to a final concentration of 150 μg/ml and the bead/protein mixture was incubated at 4°C for 24 hours with gentle rotation. The beads were washed extensively and stored at 4°C in PBS/ 0.1% BSA/ 0.01% NaN_3 at a concentration of $2x10^8$ beads/ml.

2) Alternatively, magnetic beads activated by carboxy groups can be attached to the BSA-phOx conjugate. Thus, 2 ml of 0.01 M sodium acetate buffer (pH 5.0); the phOx-BSA conjugate from above (2 mg), 2 ml of 0.45 micron carboxylpolystyrene-plated magneted beads and 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDAC, Sigma, St.

Louis, MO) were combined in a 15 ml glass centrifuge tube. The suspension was vortexed and incubated for two hours at ambient temperature on a rotary mixer. The suspension was subjected to a strong magnetic field and the supernatent was decanted. The beads were resuspended in 4 ml of the sodium acetate buffer and repelleted with the magnetic field twice to wash away contaminants.

EXAMPLE III

Transfection and Selection of Cells

This example describes methods for transfection of cells and selection with hapten capturing agent through its coupling to a cell separation means.

a. Eukaryotic Cell Transfection

Following confirmation of the integrity of the phOx sFv:PDGFRTM coding sequences, as described in Example II above, transient expression was carried out in cultured cells.

Cell lines tested include the "293" adenovirus-transformed human kidney cells, the human adenocarcinomas of the breast described in Table I, and HeLa cells, as described in above. Cell lines were grown to approximately 50-70% confluence in either RPMI-1640 or Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS, Gemini Bioproducts, Inc., Calabasas, CA) and the media

changed 24 hours prior to electroporation. Cells were harvested by incubation with trypsin or 3 mM EDTA/PBS for 5 minutes at 37°C and collected by centrifugation (800-1000 g for 5 to 10 minutes at room temperature). The supernatant was decanted. The cell pellet was then resuspended to a concentration of $1x10^7$ cells per ml in complete medium per 60 mm plate. The cells were pipetted up and down to break up cell clumps and achieve single cell suspension.

The cells, as described above, were transfected by combining 5 μg plasmid DNA with 0.2 ml cell suspension ($2x10^6$ cells) and pulsing the mixture at 500 μF and 250 V in an IBI Gene Zapper. The electroporated cells were added to 5 ml media and incubated at 37°C in a humidified CO_2 incubator. Adherent cells were harvested by incubation with PBS/ 3 mM EDTA and combined with cells that remained suspended. Cells were collected by centrifugation and resuspended in 0.5 ml medium to which $1.5x10^5$ phOx-BSA coated magnetic beads would be added.

b. Cell Separation by Magnetic Bead

Transfected cells were collected by centrifugation and resuspended in 0.5 ml PBS/3 mM EDTA medium, to which $1.5x10^5$ phOx-BSA coated magnetic beads will be added.

The magnetic beads were washed before use to remove the sodium azide. One microcentrifuge tube for each 60 mm plate of cells was set up. The magnetic bead slurry was vortexed to resuspend beads. 10 ul (1.5×10^6

beads) was added into each microcentrifuge tube. The beads were washed by adding 1 ml complete medium to each tube and mixed by inversion 3 times. The beads were pelleted with a strong magnet or magnetic stand and pipet or aspirate off medium.

The cell/bead mixture was rotated for 30 minutes at 37°C on a Dynal mixer. The bound cells were separated from the mixture by placing the tubes in a Dynal MPC-E magnetic particle concentrator. Unbound cells were drawn off and the bead pellet was washed twice by resuspension in 1 ml complete medium followed by gentle vortexing. Live unbound cells and bead-bound cells were counted by Trypan blue exclusion.

c. Evaluating sFv Produced from pPhOx.TM Displayed on the Cell Surface.

To determine whether the sFv produced from pPhOx.TM was successfully displayed on the cell surface, adenovirus-transformed human kidney cells "293" were transfected with either pPhOx.TM or psFv.MUT (which produces a truncated, inactive sFv) and returned to culture for 24 hours. The transiently transfected cell population was harvested and incubated with phOx-BSA magnetic beads for 30 minutes at 37°C in complete medium with gentle agitation. At the completion of the incubation, bead-bound cells were selected from culture by magnetic interaction. Upon microscopic inspection of the magnetic bead pellet, each selected cell was observed to have bound to it at least one and in many cases

several beads. Figure 3A shows cells at 24 hours post-transfection by electroporation, cells can be observed binding to phOx-BSA coated magnetic beads from culture. None of the cells that had been transfected with psFv.MUT were bound to beads or were selected from culture.

A time course of selection was performed in order to demonstrate the ability of the instant invention in selecting transfected cells very soon after introduction of exogenous DNA. In these experiments, "293" (adenovirus transformed human kidney) and HeLa cells were transfected with pPhOx.TM by electroporation. Aliquots of the transiently transfected cell population were incubated with phOx-BSA beads for 30 minutes at 1, 2, 4, and 8 hours post-transfection followed by selection and counting as described. These results, seen in Figure 3B, show that transiently transfected 293 cells (approximately 2.5% of the surviving population) were selected from the total population as early as 2 hours post-electroporation.

When HeLa cells were transfected in parallel reactions, display of phOx sFv sufficient for selection under these conditions occurred at 8 hours post-electroporation. From $2x10^6$ cells in the original population, $1x10^4$ transfected 293 cells were selected at 2 hours and $1x10^4$ HeLa cells were selected at 8 hours. This data is also displayed in Figure 3B.

Cell membrane expression of sFV from pPhOx.TM expression can occur in different cell types. pPhOx.TM

was introduced into several cell lines including four lines derived from carcinoma of the breast, as summarized in Table I, and adenovirus-transformed human kidney cells designated "293". Cells were selected at 24 hours post-electroporation on phOx-BSA beads and compared for selection efficiency. Under these transfection conditions, all cell lines tested displayed sFv on their membranes sufficient for selection from culture, as graphically displayed in Figure 3C and Table II. Selection efficiency varied across the cell lines tested. Increased selection efficiency can be obtained by optimizing transfection conditions for specific cells using techniques known to one skilled in the art.

Table II

Comparison of expression on phOx sFv and selection efficiencies in cell lines tranfected with pPhOx.TM

Cell Type	No. Selected	% of Live Cells Selected	% of Total Cells Selected	Mortality
MDA-MB-468	6.6×10^3	0.4%	0.3%	28%
MDA-MB-453	1.3×10^5	7.5%	6.5%	15%
MCF-7	1.8×10^4	4.8%	0.1%	81%
SK-BR-3	2.5×10^5	13.5%	12.5%	8%
293	3.1×10^4	25.9%	1.5%	94%
HeLa	6.4×10^3	5.9%	0.3%	95%

In parallel reactions, transfected cells were also incubated with magnetic beads coated with BSA alone as a

negative control. In each case incubation with BSA beads yielded selection efficiencies of less than 0.03% of the live cells present.

d. Selection Efficiency of Transfected Cells Evaluated by Immunoblot Analysis

As an indication of cell selection efficiency, immunoblot experiments were conducted using samples of transiently transfected cells selected from culture or those that remained unbound to magnetic beads. The presence of sFv in these cell populations was determined using an anti-HA epitope tag antibody 12CA5 (Boehringer Mannheim). MDA-MB-453 and SK-BR-3 cells (see Table I) transfected with pPhOx.TM, described above, were selected from culture at 24 hours post-transfection. Equivalent numbers of untransfected, transfected and selected, or non-selected cells were run on an SDS-polyacrylamide gel (Laemmli, 1970). Separated proteins were transferred to a nitrocellulose membrane and blocked in PBS/ 0.05% Tween-20/ 5% milk protein (Carnation, Los Angeles, CA) for 1 hour at room temperature. Membranes were probed with anti-HA epitope tag antibody, the 12CA5 antibody, by incubating with 12CA5 (Boehringer Mannheim) diluted to 5 μg/ml in blocking buffer for 1 hour at room temperature. The membranes were then washed with PBS/0.05% Tween-20 and incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (BioRad) diluted 1:5000 in blocking buffer for 1 hour at room temperature. Membranes were washed as above, developed using ECL reagents (Amersham) and exposed to film.

As shown in Figure 4, virtually all of the immunoreactive sFv appears in the cells that were selected from culture and only a trace of activity remained in the unselected cells. This result suggests that in the two cell lines tested, virtually all of the cells that express the sFv fusion protein are efficiently selected from culture.

e. Coexpression of phOx.TM and β-galactosidase in cotransfected cells

SK-BR-3 cells were co-transfected with pPhOx.TM and pCMVβ (Clontech) which carries the gene encoding β-galactosidase. Cells were mock transfected or transfected with either 5 μg pPhOx.TM, 5 μg pCMVβ, or 5 μg of each. A non-promoter containing plasmid was used as carrier DNA to make a total of 10 μg in each reaction. One third of each transfection reaction was plated in each chamber of a four chamber microscope slide (Nunc). Slides were incubated at 37°C for 24 hours then $1x10^5$ cpm of ^{125}I-phOx-BSA was added to each chamber and allowed to bind for 30 minutes. Slide chambers were then gently washed three times with 1 ml PBS. Cells were then fixed with 1% paraformaldehyde/0.2% glutaraldehyde for 2 minutes and incubated with the colorimetric substrate (5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 1 mM $MgCl_2$, 0.08% chlorobromo-indolyl β-D galactopyranoside, X-gal, Sigma) for β-galactosidase activity for 15 hours at 27°C. The slides were washed with PBS and the cells dehydrated by successive 5 minute washes in 50%, 75%, and 100% ethanol and air dried. They were then coated with photographic

emulsion (NTB-3, Kodak) and dried overnight. Coated slides were exposed at 4°C for four days and developed using Kodak developing solutions. In addition, 1 ml of each transfection reaction was incubated with phOx-BSA beads as described in Example III(b) above. The selected cells were then stained for β-galactosidase activity.

^{125}I-phOx-BSA was prepared by combining 100 μg BSA protein and 500 μCi Na^{125}I (Dupont/NEN, Boston, MA) to iodogen-coated tubes using the manufacturer's protocol (Pierce). Free ^{125}I was removed by applying reactions to an Econo-Pac 10DG column (BioRad) that had been blocked with BSA and equilibrated in PBS. Labeled protein was eluted in PBS.

The results, depicted in the radiograph/photograph of Figure 5 A-D, demonstrate that most if not all of the cells expressing the functional pPhOx.TM product (cells with silver grains, denoted by arrows) are also expressing β-galactosidase (blue staining, the point of the triangles opposite the stars points towards representative cells staining for β-galactosidase). The data demonstrates that greater than 98% of the cells selected with phOx-BSA-coated magnetic beads stained positively for β-galactosidase activity.

EXAMPLE IV

GENERAL PROCEDURE FOR CO-TRANSFECTION WITH PhOx.TM VECTOR AND SECOND PLASMID CONTAINING GENE OF INTEREST

A. Plasmid Preparation

In order to insure that the plasmid DNA used in the instant procedure is of high quality and free of contaminants, the PhOx.TM vector and the vector containing the gene of interest was subjected to CsCl gradient ultracentrifugation. Boiled or alkaline lysis miniprep DNA should not be used in this procedure. Further purification methods can be found in Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., Struhl, K., eds (1990) Current Protocols in Molecular Biology. Greene Publishing Associates and Wiley-Interscience, New York.

In addition, the PhOx.TM Vector can be amplified prior to use in the instant invention by transforming the plasmid into a *recA*, *endA* *E. coli* (e.g. DH5α) strain. The lyophilized vector is resuspended in 20 μl of sterile water to make a stock solution. A small portion (1 μl) of the stock solution can be used to transfect the *E. coli* of choice on LB plates containing 100 μg/ml ampicillin or 50 μg/ml kanamycin.

B. Positive Control

The pCR™3*lacZ* (8.1 kb) plasmid used in this procedure as a positive control is constructed by inserting the *lacZ* gene in the EcoR1 cite of the pCR™3 plasmid (Invitrogen, San Diego, CA). The positive control serves to assist in optimizing the transfection conditions for

the PhOx.TM and co-tranfected vectors. The pCR™3*lacZ* contains the *E. coli* gene encoding β-galactosidase, which gene is expressed in mammalian cells using the immediate-early promoter from cytomegalovirus. A successful cotransfection with the PhOx.TM or the vector bearing the gene of interest will result in positive β-galactosidase expression in selected cells and can be easily monitored with a colorimetric b-galactosidase assay, as described below.

C. Methods of Transfection

Transfection procedures for the cell line of interest may often be found in articles discussing that particular cell line. Such methods of transfections are well known and may include calcium phosphate, DEAE-dextran, liposome-mediated, or electroporation. The protocol discussed in the art for the cell line of interest should be followed exactly. Particular attention should be paid to medium requirements, when to pass the cells, and at what dilution to split the cells. Further information can be found in Current Protocols in Molecular Biology, supra.

In the event that the art does not teach a transfection method for the cell line of interest, electroporation is the method of choice. For instance, the following electroporation protocol may be used (a "no DNA" negative control should also be used):

1. Prepare Trypsin/versene (EDTA) or PBS/3 mM EDTA. The latter can be prepared as follows:

137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
1.8 mM KH_2PO_4
(3 mM EDTA, optional)

a. Dissolve: 8 g NaCl
0.2 g KCl
1.44 g Na_2HPO_4
0.24 g KH_2PO_4
(6 ml 0.5 M EDTA, pH 8)
in 800 ml deionized water.

b. Adjust the pH to 7.4 with concentrated HCl.

c. Bring the volume to 1 liter and autoclave for 20 minutes on liquid cycle.

d. Store at +4°C or room temperature.

2. Change medium on the cells 24 hours prior to electroporation.

3. Harvest the cells at 60-80% confluency using half of the initial culture volume of PBS/3 mM EDTA.

4. Count the cells and resuspend them in complete medium at 1 x 10^7 cells /ml.

5. Mix PhOx.TM and the construct containing the gene of interest (or pCR-3*lacZ*) in a 1:1 molar

ratio in a volume of 10 µl or less. Use 1-5 µg of each plasmid.

6. The plasmid mixture is added to 200 µl of the cell suspension (2×10^6 cells). The suspension is mixed gently and is transferred to a chilled electroporation cuvette (0.4 cm gap width).

7. The cells are electroporated using the recommended settings of the electroporation device.

8. The electroporated cells are transferred to a 60mm plate containing 5-7 ml complete medium. The plates are incubated in a 37°C, 5% CO_2 incubator for 2-48 hours.

D. Cell Selection

The transfected cells from the above Section C can be isolated using the following procedure. In general, the procedure employs 1.5×10^6 beads per 60 mm plate of transfected cells. These conditions may vary due to the method of transfection and the cell line used. Sterile techniques should be used when performing the following steps.

1. Preparation of Transfected Cells

The PBS/3 mM EDTA buffer described above and complete medium should be prepared before attempting the following steps:

a. PBS/3 mM EDTA (3-5 ml) is added to the cells. The cells are incubated for 5 minutes at 37°C and then are harvested. Untransfected cells (or the cells from the negative transfection control) may be harvested for use as a negative control when assaying for b-galactosidase activity.

b. The cells are centrifuged at 800-1000 x g for 5-10 minutes at room temperature. The supernatant is decanted.

c. The cells are resuspended in 1 ml complete medium per 60 mm plate. The cells are pipetted up and down in order to break up cell clumps and achieve a single-cell suspension.

2. Preparation of Magnetic Beads

The magnetic beads are washed before use to remove any sodium azide present.

d. A microcentrifuge tube is prepared for each 60 mm plate of cells.

e. The magnetic beads slurry is vortexed to resuspend beads and is added (10 μl (1.5 x 10^6 beads)) into each microcentrifuge tube.

f. The beads are washed by adding 1 ml complete medium to each tube and are mixed by inversion 3 times. The beads are pelleted with a strong

magnet or magnetic stand and the medium is removed by pipetting or aspiration.

3. Selection of Transfected Cells

g. Cell suspension (1 ml) from Step 1C is added to a tube containing washed beads from Step 2f. The suspension is incubated for 30 minutes.

h. The tubes containing the bead-cell mixture are placed in a magnetic stand and are mixed for 30 seconds to 1 minute with a gentle end over end rotation.

i. While the tube is still in contact with the magnet, the non-selected cells are removed with a pipet. (These cells may be saved for further analysis.)

j. The tubes are removed from the magnetic stand and the beads and cells are resuspended in 1 ml complete medium. The suspension is vortexed gently.

k. The beads (and bound cells) are pelleted using the magnetic stand, the supernatant is removed by pipet.

l. Repeat Steps j and k two more times.

m. Selected cells are resuspended in 100 µl complete medium (for pCR™3*lacZ* control, use X-gal Reagent, see below) and the cells are counted. The cells are ready to culture or analyze.

E. Optimization of Cell Transfection

The first step in utilizing the method of this invention can be to optimize the transfection conditions for the cell line of interest. Once transfection conditions have been optimized, the cell line can then be cotransfected with the PhOx.TM vector and the vector containing the gene of interest.

The pCR™3*lacZ* positive control plasmid can be used to check for cotransfection of selected cells and assessing transfection efficiencies. Transfected cells are selected using the above methods. Untransfected cells, selected cells, and non-selected cells are assayed with X-gal and counted. (Cells expressing b-galactosidase will turn blue in the presence of X-gal.) Comparison of the number of blue, non-selected cells versus blue, selected cells will allow the determinination of selection efficiency. (Untransfected cells should not stain with X-gal.) Optimal cotransfection conditions are defined as when the PhOx.TM to pCR™3*lacZ* ratio gives the greatest enrichment of blue-stained cells in the selected population.

1. Preparation of X-gal Reagent

1 mg/ml X-Gal in DMF
4 mM potassium ferricyanide ($K_3Fe(CN)_6$)
4 mM potassium ferrocyanide ($K_4Fe(CN)_6$-$3H_2O$)
2 mM magnesium chloride hexahydrate
in PBS, pH 7.4

a. Each of the following stock solutions (10 ml) are prepared. These solutions are stable indefinitely if stored as indicated.

- X-gal: (20 mg/ml in dimethylformamide (DMF)): Dissolve 200 mg of X-gal in 10 ml DMF and store at -20°C.
- Potassium Ferricyanide and Potassium Ferrocyanide: (0.4 M each in deionized water.): Dissolve 1.32 g of potassium ferricyanide and 1.69 g of potassium ferrocyanide in 10 ml deionized water. Store at -20°C.
- Magnesium Chloride: (200 mM in deionized water.): Dissolve 0.4 g in 10 ml deionized water and store at room temperature or -20°C.

b. For 10 ml of X-gal reagent, mix together:
0.5 ml of 20 mg/ml X-Gal stock solution;
0.1 ml of the potassium ferricyanide/ferrocyanide stock solution;
0.1 ml of the magnesium chloride stock solution; and
9.3 ml of PBS.

2. Colorimetric Assay for β-galactosidase

a. To assay selected cells:

i. The selected cells are resuspended in 100 µl X-gal Reagent:

ii. The cells are incubated overnight at room temperature:

iii. The cells are examined under the microscope for the development of blue color and the number of stained and total cells is counted.

b. To assay non-selected cells:

i. The non-selected cells are centrifuged 5 minutes at 4000 rpm to pellet the cells. The supernatant is decanted.

ii. The cells are resuspended in 1 ml PBS and again pelleted. The supernatant is decanted.

iii. The cells are resuspended in 100 µl of X-gal Reagent and are incubated overnight at room temperature.

iv. The cells are examined under a microscope for the development of blue color. The number of total cells and blue cells are counted.

c. To assay untransfected cells (negative control):

i. The untransfected cells are centrifuged for 5 minutes at 4000 rpm to pellet the cells.

ii. The cells are resuspended in 1 ml PBS and recentrifuged in order to pellet the cells.

iii. The cells are resuspended in 100 µl of X-gal Reagent and are incubated overnight at room temperature.

iv. The cells are examined under a microscope for the development of blue color. The number of total cells and blue cells are counted.

In all of the above counting procedures the total cell number is normalized.

F. Optimization of Cell Selection

The presence of unbound beads after the application of the magnet to the transfection mixture indicates that a proper number of magnetic beads. If no unbound beads are observed, it may mean that not all tranfected cells were selected in the procedure. Should the procedure using those particular conditions be repeated, it is desirable to double the number of beads (e.g., 20 μl or 3 x 10^6 beads) in order to ensure that you isolate all transfected cells.

In the transfection optimization procedure, nearly all selected cells should express β-galactosidase. If there are non-selected cells that are blue, then the relative amount of PhOx.TM to pCR™3*lac*Z should be increased.

Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made by those skilled in the art without departing from the

invention. Accordingly, the invention is set out in the following claims.

WE CLAIM:

1. A eukaryotic expression vector for the identification and separation of transfected cells from a total cell population, comprising:

a first DNA sequence encoding an anti-hapten recombinant antibody, said recombinant antibody capable of binding a specific hapten;

a second DNA sequence encoding for a transmembrane domain functionally linked to said first DNA sequence;

a third DNA sequence encoding for a signal sequence functionally linked to said first DNA sequence;

a first promoter operatively linked to said first DNA sequence;

at least one additional DNA sequence encoding for at least one protein;

a promoter operatively linked to said additional DNA sequence.

2. The eukaryotic expression vector of claim 1, wherein said first DNA sequence encodes a single-chained, hapten-binding antibody.

3. The eukaryotic expression vector of claim 1, wherein said hapten is 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one.

4. The eukaryotic expression vector of claim 1, wherein said vector is selected from the group consisting of a plasmid, a virus, or linear double-stranded DNA.

5. The eukaryotic expression vector of claim 1, wherein said transmembrane domain comprises an immunoglobulin or a platelet-derived growth factor transmembrane domain.

6. The eukaryotic expression vector of claim 1, wherein said signal sequence comprises the murine immunoglobulin kappa chain V-J2-C region signal peptide.

7. The eukaryotic expression vector of claim 1, wherein said first promoter is selected from the group consisting of cytomegalovirus (CMV) immediate early promoter, Rous sarcoma virus (RSV) promoter, adenovirus major late promoter, SV40 early promoter and retroviral long terminal repeats (LTRs).

8. The eukaryotic expression vector of claim 1, wherein said recombinant antibody is expressed extracellularly at least two hours after transfection.

9. The eukaryotic expression vector of claim 1, wherein the expression of the protein encoded by said fourth DNA sequence affects the physiology of the eukaryotic cell.

10. A eukaryotic cell transfected with the eukaryotic expression vector of claim 1.

11. A mixture of eukaryotic expression vectors for the identification and separation of transfected cells from a total cell population, comprising a first vector which in turn comprises:

a first DNA sequence encoding an anti-hapten recombinant antibody, said recombinant antibody capable of binding a specific hapten;

a second DNA sequence encoding for a transmembrane domain functionally linked to said first DNA sequence;

a third DNA sequence encoding for a signal sequence functionally linked to said first DNA sequence;

a promoter operatively linked to said first DNA sequence;

at least one additional vector encoding for at least one protein.

12. The eukaryotic expression vector of claim 11, wherein said first DNA sequence encodes a single-chained, hapten-binding antibody.

13. The eukaryotic expression vector of claim 11, wherein said hapten is 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one.

14. The eukaryotic expression vector of claim 11, wherein said vector is selected from the group consisting of a plasmid, a virus, or linear double-stranded DNA.

15. The eukaryotic expression vector of claim 11, wherein said transmembrane domain comprises an immunoglobulin or a platelet-derived growth factor transmembrane domain.

16. The eukaryotic expression vector of claim 11, wherein said signal sequence comprises the murine immunoglobulin kappa chain V-J2-C region signal peptide.

17. The eukaryotic expression vector of claim 11, wherein said promoter is selected from the group consisting of cytomegalovirus (CMV) immediate early promoter, Rous sarcoma virus (RSV) promoter, adenovirus major late promoter, SV40 early promoter and viral long terminal repeats (LTRs).

18. The eukaryotic expression vector of claim 11, wherein said recombinant antibody is expressed extracellularly at least two hours after transfection.

19. A eukaryotic cell transfected with the eukaryotic expression vector of claim 11.

20. A method of identifying and isolating transfected cells from the total cell population, comprising:

transfecting a eukaryotic cell with the eukaryotic expression vector of claim 1;

exposing said cell to a hapten conjugated to a cell selection means;

separating said cell, bound to said selection means, from the total cell population.

21. The method of claim 20, wherein said first DNA coding sequence comprises a sequence encoding a single-chained, hapten-binding antibody.

22. The method of claim 20, wherein said hapten is 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one.

23. The method of claim 20, wherein said vector is selected from the group consisting of a plasmid, a virus or double-stranded DNA.

24. The method of claim 20, wherein said transmembrane domain comprises an immunoglobulin or a platelet derived growth factor transmembrane domain.

25. The method of claim 20, wherein said signal sequence comprises a murine immunoglobulin kappa chain V-J2-C region signal peptide.

26. The method of claim 20, wherein said first promoter comprises cytomegalovirus (CMV) immediate early promoter, Rous sarcoma virus (RSV) promoter, adenovirus major late promoter, SV40 early promoter or retroviral long terminal repeats (LTRs).

27. The method of claim 20, wherein said recombinant antibody is expressed extracellularly at least two hours after transfection.

28. The method of claim 20, wherein said transfecting of said cell is effected by electroporation.

29. The method of claim 20, wherein said separating of said cell is effected by physical separation.

30. The method of claim 20, wherein said cell separation means comprises magnetic beads.

31. A method of identifying and isolating transfected cells from the total cell population, comprising:

transfecting a eukaryotic cell with the eukaryotic expression vector of claim 11;

exposing said cell to a hapten conjugated to a cell selection means;

separating said cell, bound to said selection means, from the total cell population.

32. The method of claim 31, wherein said first DNA coding sequence comprises a sequence encoding a single-chained, hapten-binding antibody.

33. The method of claim 31, wherein said hapten is 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one.

34. The method of claim 31, wherein said vector is selected from the group consisting of a plasmid, a virus or double-stranded DNA.

35. The method of claim 31, wherein said transmembrane domain comprises an immunoglobulin or a platelet derived growth factor transmembrane domain.

36. The method of claim 31, wherein said signal sequence comprises a murine immunoglobulin kappa chain V-J2-C region signal peptide.

37. The method of claim 31, wherein said promoter comprises cytomegalovirus (CMV) immediate early promoter, Rous sarcoma virus (RSV) promoter, adenovirus major late promoter, SV40 early promoter or viral long terminal repeats (LTRs).

38. The method of claim 31, wherein said recombinant antibody is expressed extracellularly at least two hours after transfection.

39. The method of claim 31, wherein said transfecting of said cell is effected by electroporation.

40. The method of claim 31, wherein said separating of said cell is effected by physical separation.

41. The method of claim 31, wherein said cell separation means comprises magnetic beads.

42. A kit for the identification and separation of transfected cells from a total cell population, comprising:

the eukaryotic expression vector of claim 1;

a cell separation means.

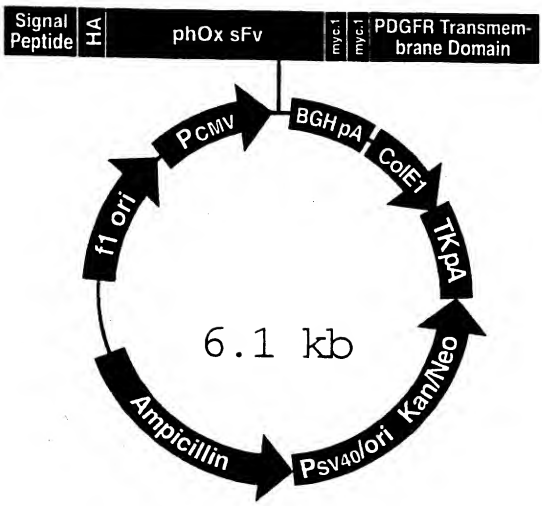
43. The kit of claim 42, wherein said cell separation means comprises magnetic beads.

44. The kit of claim 43, wherein said cell separation means further comprises magnetic beads coated with a hapten.

45. The kit of claim 44, wherein said hapten comprises 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one.

Feature	Benefit
PhOx sFv	This single chain antibody recognizes the hapten, phOx and allows isolation or detection of cells displaying this sFv (Griffiths, *et al.*, 1984; Hoogenboom, *et al.*, 1991)
Cytomegalovirus (CMV) immediate early promoter	Permits high-level expression of the sFv in a wide variety of eukaryotic cells
Signal peptide (Met-Glu-Thr-Asp-Thr-Leu-Leu-Leu-Trp-Val-Leu-Leu-Leu-Trp-Val-Pro-Gly-Ser-Thr-Gly-Asp)	Signal peptide from murine Ig κ-chain V-J2-C region directs the sFv to the plasma membrane for extracellular display
Hemagglutinin A epitope tag (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala)	Allows detection of the sFv by monoclonal antibody 12CA5 (Kolodziej and Young, 1991; Niman, *et al.*, 1983)
Myc.1 epitope tag (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Asn)	Allows detection of the sFv by the monoclonal antibody 9E10.2 (Evan, *et al.*, 1985)
Platelet-derived growth factor receptor transmembrane domain (PDGFR-TM)	Fusion of PDGFR-TM to sFv anchors the antibody to the plasma membrane for display
Bovine growth hormone polyadenylation signal	Permits proper processing and polyadenylation of the mRNA for stabilization of the message
Ampicillin resistance gene	Allows selection of the plasmid in *E. coli*
ColE1 origin	High copy replication and growth in *E. coli*
Kanamycin resistance gene	Allows selection of the plasmid in *E. coli* using kanamycin Note: this gene will also confer resistance to G418 in mammalian cells
SV40 promoter and origin	Permits expression of the kanamycin resistance gene for G418 resistance in mammalian cells Allows episomal replication in cells containing SV40 large T antigen

FIG. 1A-1



Comments for pHook™-1:
6115 nucleotides

CMV promoter: bases 1-596
Murine Ig kappa-chain V-J2-C signal peptide: bases 737-799
Hemagglutinin A epitope: bases 800-826
phOx sFv: bases 842-1555
Myc.1 epitope 1: bases 1568-1600
Myc.1 epitope 2: bases 1613-1645
PDGFR transmembrane domain: bases 1646-1795
Bovine growth hormone polyadenylation signal: bases 1853-2081
Col E1 origin: bases 2212-2795
SV40 origin and promoter: bases 4587-4249
Neomycin/Kanamycin resistance gene: bases 4214-3426
Thymidine kinase polyadenylation site: bases 3251-2980
Ampicillin resistance gene: bases 55526-4666
f1 origin: bases 5657-6113

FIG. 1A-2

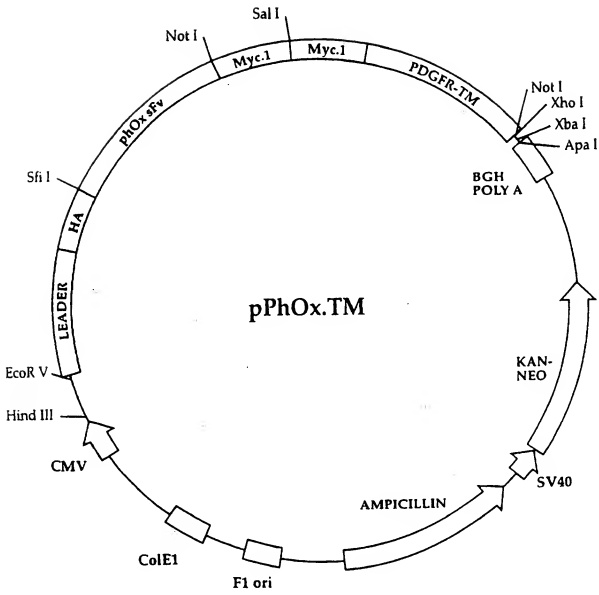


FIG. 1B

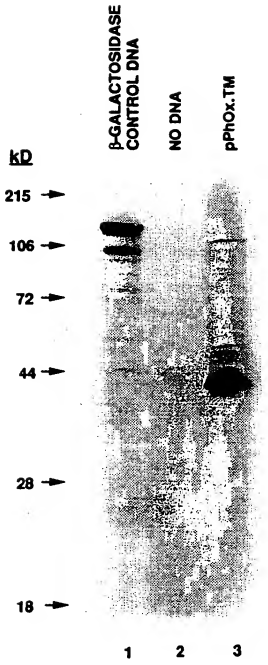


FIG. 2

FIG. 3A

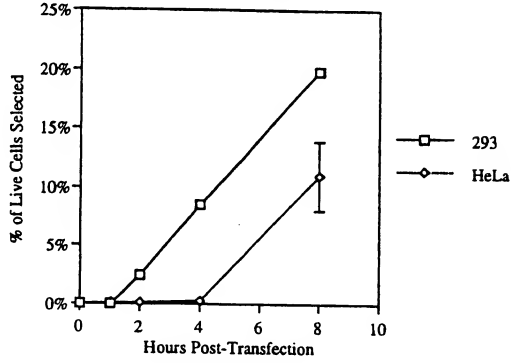


FIG. 3B

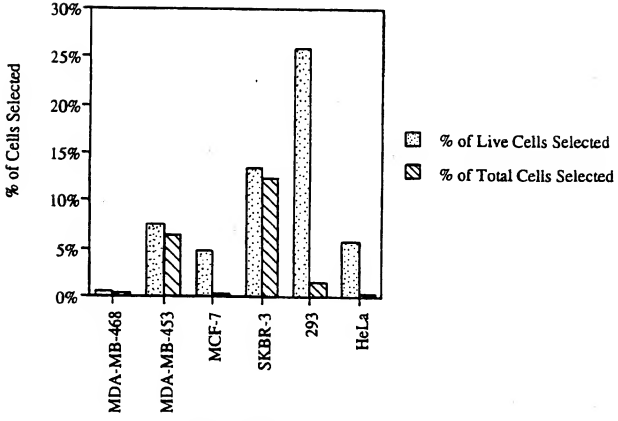


FIG. 3C

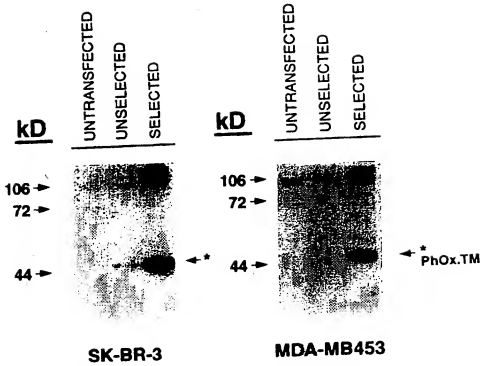


FIG. 4A

FIG. 4B

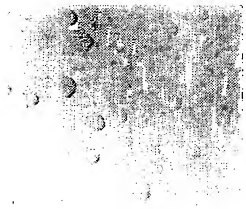


FIG. 5A

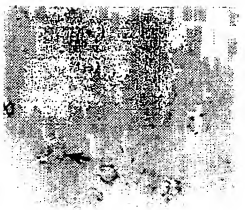


FIG. 5B

FIG. 5C

FIG. 5D

CMV promoter: bases 1-596
T7 promoter: bases 638-657

Murine Ig kappa-chain V-J2-C signal peptide: bases 737-799
Hemagglutinin A epitope: bases 800-826
phOx sFv: bases 842-1555
Myc.1 epitope 1: bases 1568-1600
Myc.1 epitope 2: bases 1613-1645
PDGFR transmembrane domain: bases 1646-1795

SP6 promoter: bases 1831-1848
Bovine growth hormone polyadenylation signal: bases 1853-2081

Col E1 origin: bases 2212-2795

SV40 origin and promoter: bases 4587-4249
Neomycin/Kanamycin resistance gene: bases 4214-3426
Thymidine kinase polyadenylation site: bases 3251-2980

Ampicillin resistance gene: bases 5526-4666
f1 origin: bases 5657-6113

```
          10         20         30         40         50         60
GCGCGCGTTG ACATTGATTA TTGACTAGTT ATTAATAGTA ATCAATTACG GGGTCATTAG
CGCGCGCAAC TGTAACTAAT AACTGATCAA TAATTATCAT TAGTTAATGC CCCAGTAATC

          70         80         90        100        110        120
TTCATAGCCC ATATATGGAG TTCCGCGTTA CATAACTTAC GGTAAATGGC CCGCCTGGCT
AAGTATCGGG TATATACCTC AAGGCGCAAT GTATTGAATG CCATTTACCG GGCGGACCGA

          130        140        150        160        170        180
GACCGCCCAA CGACCCCCGC CCATTGACGT CAATAATGAC GTATGTTCCC ATAGTAACGC
CTGGCGGGTT GCTGGGGGCG GGTAACTGCA GTTATTACTG CATACAAGGG TATCATTGCG
```

FIG. 6A

```
       190        200        210        220        230        240
CAATAGGGAC TTTCCATTGA CGTCAATGGG TGGACTATTT ACGGTAAACT GCCCACTTGG
GTTATCCCTG AAAGGTAACT GCAGTTACCC ACCTGATAAA TGCCATTTGA CGGGTGAACC

       250        260        270        280        290        300
CAGTACATCA AGTGTATCAT ATGCCAAGTA CGCCCCCTAT TGACGTCAAT GACGGTAAAT
GTCATGTAGT TCACATAGTA TACGGTTCAT GCGGGGGATA ACTGCAGTTA CTGCCATTTA

       310        320        330        340        350        360
GGCCCGCCTG GCATTATGCC CAGTACATGA CCTTATGGGA CTTTCCTACT TGGCAGTACA
CCGGGCGGAC CGTAATACGG GTCATGTACT GGAATACCCT GAAAGGATGA ACCGTCATGT

       370        380        390        400        410        420
TCTACGTATT AGTCATCGCT ATTACCATGG TGATGCGGTT TTGGCAGTAC ATCAATGGGC
AGATGCATAA TCAGTAGCGA TAATGGTACC ACTACGCCAA AACCGTCATG TAGTTACCCG

       430        440        450        460        470        480
GTGGATAGCG GTTTGACTCA CGGGGATTTC CAAGTCTCCA CCCCATTGAC GTCAATGGGA
CACCTATCGC CAAACTGAGT GCCCCTAAAG GTTCAGAGGT GGGGTAACTG CAGTTACCCT

       490        500        510        520        530        540
GTTTGTTTTG GCACCAAAAT CAACGGGACT TTCCAAAATG TCGTAACAAC TCCGCCCCAT
CAAACAAAAC CGTGGTTTTA GTTGCCCTGA AAGGTTTTAC AGCATTGTTG AGGCGGGGTA

       550        560        570        580        590        600
TGACGCAAAT GGGCGGTAGG CGTGTACGGT GGGAGGTCTA TATAAGCAGA GCTCTCTGGC
ACTGCGTTTA CCCGCCATCC GCACATGCCA CCCTCCAGAT ATATTCGTCT CGAGAGACCG

       610        620        630        640        650        660
```

FIG. 6B

```
TAACTAGAGA ACCCACTGCT TACTGGCTTA TCGAAATTAA TACGACTCAC TATAGGGAGA
ATTGATCTCT TGGGTGACGA ATGACCGAAT AGCTTTAATT ATGCTGAGTG ATATCCCTCT

       670        680        690        700        710        720
CCCAAGCTTG GTACCGAGCT CGGATCCACT AGTAACGGCC GCCAGTGTGC TGGAATTCGG
GGGTTCGAAC CATGGCTCGA GCCTAGGTGA TCATTGCCGG CGGTCACACG ACCTTAAGCC

       730        740        750        760        770        780
CTTGGGGATA TCCACCATGG AGACAGACAC ACTCCTGCTA TGGGTACTGC TGCTCTGGGT
GAACCCCTAT AGGTGGTACC TCTGTCTGTG TGAGGACGAT ACCCATGACG ACGAGACCCA

       790        800        810        820        830        840
TCCAGGTTCC ACTGGTGACT ATCCATATGA TGTTCCAGAT TATGCTGGGG CCCAGCCGGC
AGGTCCAAGG TGACCACTGA TAGGTATACT ACAAGGTCTA ATACGACCCC GGGTCGGCCG

       850        860        870        880        890        900
CATGGCCGAG GTCAAGCTGC AGGAGTCAGG GGGAGGCTTA GTGCAGCCTG GAGGGTCCCG
GTACCGGCTC CAGTTCGACG TCCTCAGTCC CCCTCCGAAT CACGTCGGAC CTCCCAGGGC

       910        920        930        940        950        960
GAAACTCTCC TGTGCAGCCT CTGGATTCAC TTTCAGTAGC TTTGGAATGC ACTGGGTTCG
CTTTGAGAGG ACACGTCGGA GACCTAAGTG AAAGTCATCG AAACCTTACG TGACCCAAGC

       970        980        990       1000       1010       1020
TCAGGCTCCA GAGAAGGGGC TGGAGTGGGT CGCATATATT AGTAGTGGCA GTAGTACCAT
AGTCCGAGGT CTCTTCCCCG ACCTCACCCA GCGTATATAA TCATCACCGT CATCATGGTA

       1030       1040       1050       1060       1070       1080
CTACTATGCA GACACAGTGA AGGGACGATT CACCATCTCC AGAGACAATC CCAAGAACAC
GATGATACGT CTGTGTCACT TCCCTGCTAA GTGGTAGAGG TCTCTGTTAG GGTTCTTGTG
```

FIG. 6C

```
1090        1100        1110        1120        1130        1140
CCTGTTCCTG  CAAATGACCA  GTCTAAGGTC  TGAGGACACG  GNCATGTATT  ACTGTGCAAG
GGACAAGGAC  GTTTACTGGT  CAGATTCCAG  ACTCCTGTGC  CNGTACATAA  TGACACGTTC

1150        1160        1170        1180        1190        1200
AGATTACGGG  GCTTATTGGG  GCCAAGGGAC  CACGGNCACC  GTCTCCTCAG  GTGGAGCGCG
TCTAATGCCC  CGAATAACCC  CGGTTCCCTG  GTGCCNGTGG  CAGAGGAGTC  CACCTCCGCC

1210        1220        1230        1240        1250        1260
CTCAGCGCGA  GGTGGCTCTG  GCGGTGGCGG  ATCGGACATT  GAGCTCACCC  AGTCTCCAGC
GAGTCCGCCT  CCACCGAGAC  CGCCACCGCC  TAGCCTGTAA  CTCGAGTGGG  TCAGAGGTCG

1270        1280        1290        1300        1310        1320
AATCATGTCT  GCATCTCCAG  GGGAGAGGGT  CACCATGACC  TGCAGTGCCA  GTTCAAGTGT
TTAGTACAGA  CGTAGAGGTC  CCCTCTCCCA  GTGGTACTGG  ACGTCACGGT  CAAGTTCACA

1330        1340        1350        1360        1370        1380
AAGGTACATG  AACTGGTTCC  AACAGAAGTC  AGGCACCTCC  CCCAAAAGAT  GGATTTATGA
TTCCATGTAC  TTGACCAAGG  TTGTCTTCAG  TCCGTGGAGG  GGGTTTTCTA  CCTAAATACT

1390        1400        1410        1420        1430        1440
CACATCCAAA  CTGTCTTCTG  GAGTCCCTGC  TCGCTTCAGT  GGCAGTGGGT  CTGGGACCTC
GTGTAGGTTT  GACAGAAGAC  CTCAGGGACG  AGCGAAGTCA  CCGTCACCCA  GACCCTGGAG

1450        1460        1470        1480        1490        1500
TTACTCTCTC  ACAATCAGCA  GCATGGAGGC  TGAAGATGCT  GCCACTTACT  ACTGCCAGCA
AATGAGAGAG  TGTTAGTCGT  CGTACCTCCG  ACTTCTACGA  CGGTGAATGA  TGACGGTCGT

1510        1520        1530        1540        1550        1560
GTGGAGTAGT  AACCCACTCA  CGTTCGGTGC  TGGGACCAAG  CTGGAGCTGA  AACGG---GC
CACCTCATCA  TTGGGTGAGT  GCAAGCCACG  ACCCTGGTTC  GACCTCGACT  TTGCC---CG
```

FIG. 6D

```
      1570       1580       1590       1600       1610       1620
GGCCGCAGAA CAAAAACTCA TCTCAGAAGA GGATCTGAAT GGGGCCGTCG ACGAACAAAA
CCGGCGTCTT GTTTTTGAGT AGAGTCTTCT CCTAGACTTA CCCCGGCAGC TGCTTGTTTT

      1630       1640       1650       1660       1670       1680
ACTCATCTCA GAAGAGGATC TGAATGCTGT GGGCCAGGAC ACGCAGGAGG TCATCGTGGT
TGAGTAGAGT CTTCTCCTAG ACTTACGACA CCCGGTCCTG TGCGTCCTCC AGTAGCACCA

      1690       1700       1710       1720       1730       1740
GCCACACTCC TTGCCCTTTA AGGTGGTGGT GATCTCAGCC ATCCTGGCCC TGGTGGTGCT
CGGTGTGAGG AACGGGAAAT TCCACCACCA CTAGAGTCGG TAGGACCGGG ACCACCACGA

      1750       1760       1770       1780       1790       1800
CACCATCATC TCCCTTATCA TCCTCATCAT GCTTTGGCAG AAGAAGCCAC GTTAGGCGGC
GTGGTAGTAG AGGGAATAGT AGGAGTAGTA CGAAACCGTC TTCTTCGGTG CAATCCGCCG

      1810       1820       1830       1840       1850       1860
CGCTCGAGCA TGCATCTAGA GGGCCCTATT CTATAGTGTC ACCTAAATGC TAGAGCTCGC
GCGAGCTCGT ACGTAGATCT CCCGGGATAA GATATCACAG TGGATTTACG ATCTCGAGCG

      1870       1880       1890       1900       1910       1920
TGATCAGCCT CGACTGTGCC TTCTAGTTGC CAGCCATCTG TTGTTTGCCC CTCCCCCGTG
ACTAGTCGGA GCTGACACGG AAGATCAACG GTCGGTAGAC AACAAACGGG GAGGGGGCAC

      1930       1940       1950       1960       1970       1980
CCTTCCTTGA CCCTGGAAGG TGCCACTCCC ACTGTCCTTT CCTAATAAAA TGAGGAAATT
GGAAGGAACT GGGACCTTCC ACGGTGAGGG TGACAGGAAA GGATTATTTT ACTCCTTTAA

      1990       2000       2010       2020       2030       2040
GCATCGCATT GTCTGAGTAG GTGTCATTCT ATTCTGGGGG GTGGGGTGGG GCAGGACAGC
CGTAGCGTAA CAGACTCATC CACAGTAAGA TAAGACCCCC CACCCCACCC CGTCCTGTCG
```

FIG. 6E

```
                2050        2060        2070        2080        2090        2100
AAGGGGGAGG ATTGGGAAGA CAATAGCAGG CATGCTGGGG ATGCGGTGGG CTCTATGGCT
TTCCCCCTCC TAACCCTTCT GTTATCGTCC GTACGACCCC TACGCCACCC GAGATACCGA

                2110        2120        2130        2140        2150        2160
TCTGAGGCGG AAAGAACCAG TGGCGGTAAT ACGGTTATCC ACAGAATCAG GGGATAACGC
AGACTCCGCC TTTCTTGGTC ACCGCCATTA TGCCAATAGG TGTCTTTAGTC CCCTATTGCG

                2170        2180        2190        2200        2210        2220
AGGAAAGAAC ATGTGAGCAA AAGGCCAGCA AAAGGCCAGG AACCGTAAAA AGGCCGCGTT
TCCTTTCTTG TACACTCGTT TTCCGGTCGT TTTCCGGTCC TTGGCATTTT TCCGGCGCAA

                2230        2240        2250        2260        2270        2280
GCTGGCGTTT TTCCATAGGC TCCGCCCCCC TGACGAGCAT CACAAAAATC GACGCTCAAG
CGACCGCAAA AAGGTATCCG AGGCGGGGGG ACTGCTCGTA GTGTTTTTAG CTGCGAGTTC

                2290        2300        2310        2320        2330        2340
TCAGAGGTGG CGAAACCCGA CAGGACTATA AAGATACCAG GCGTTTCCCC CTGGAAGCTC
AGTCTCCACC GCTTTGGGCT GTCCTGATAT TTCTATGGTC CGCAAAGGGG GACCTTCGAG

                2350        2360        2370        2380        2390        2400
CCTCGTGCGC TCTCCTGTTC CGACCCTGCC GCTTACCGGA TACCTGTCCG CCTTTCTCCC
GGAGCACGCG AGAGGACAAG GCTGGGACGG CGAATGGCCT ATGGACAGGC GGAAAGAGGG

                2410        2420        2430        2440        2450        2460
TTCGGGAAGC GTGGCGCTTT CTCATAGCTC ACGCTGTAGG TATCTCAGTT CGGTGTAGGT
AAGCCCTTCG CACCGCGAAA GAGTATCGAG TGCGACATCC ATAGAGTCAA GCCACATCCA

                2470        2480        2490        2500        2510        2520
CGTTCGCTCC AAGCTGGGCT GTGTGCACGA ACCCCCCGTT CAGCCCGACC GCTGCGCCTT
GCAAGCGAGG TTCGACCCGA CACACGTGCT TGGGGGGCAA GTCGGGCTGG CGACGCGGAA
```

FIG. 6F

```
2530        2540        2550        2560        2570        2580
ATCCGGTAAC TATCGTCTTG AGTCCAACCC GGTAAGACAC GACTTATCGC CACTGGCAGC
TAGGCCATTG ATAGCAGAAC TCAGGTTGGG CCATTCTGTG CTGAATAGCG GTGACCGTCG

2590        2600        2610        2620        2630        2640
AGCCACTGGT AACAGGATTA GCAGAGCGAG GTATGTAGGC GGTGCTACAG AGTTCTTGAA
TCGGTGACCA TTGTCCTAAT CGTCTCGCTC CATACATCCG CCACGATGTC TCAAGAACTT

2650        2660        2670        2680        2690        2700
GTGGTGGCCT AACTACGGCT ACACTAGAAG GACAGTATTT GGTATCTGCG CTCTGCTGAA
CACCACCGGA TTGATGCCGA TGTGATCTTC CTGTCATAAA CCATAGACGC GAGACGACTT

2710        2720        2730        2740        2750        2760
GCCAGTTACC TTCGGAAAAA GAGTTGGTAG CTCTTGATCC GGCAAACAAA CCACCGCTGG
CGGTCAATGG AAGCCTTTTT CTCAACCATC GAGAACTAGG CCGTTTGTTT GGTGGCGACC

2770        2780        2790        2800        2810        2820
TAGCGGTGGT TTTTTTGTTT GCAAGCAGCA GATTACGCGC AGAAAAAAAG GATCTCAAGA
ATCGCCACCA AAAAAACAAA CGTTCGTCGT CTAATGCGCG TCTTTTTTTC CTAGAGTTCT

2830        2840        2850        2860        2870        2880
AGATCCTTTG ATCTTTTCTA CGGGGTCTGA CGCTCAGTGG AACGAAAACT CACGTTAAGG
TCTAGGAAAC TAGAAAAGAT GCCCCAGACT GCGAGTCACC TTGCTTTTGA GTGCAATTCC

2890        2900        2910        2920        2930        2940
GATTTTGGTC ATGAGATTAT CAAAAAGGAT CTTCACCTAG ATCCTTTTAA ATTAAAAATG
CTAAAACCAG TACTCTAATA GTTTTTCCTA GAAGTGGATC TAGGAAAATT TAATTTTTAC

2950        2960        2970        2980        2990        3000
AAGTTTTAAA TCAATCTAAA GTATATATGA GTAACCTGAG GCTATGGCAG GGCCTGCCGC
TTCAAAATTT AGTTAGATTT CATATATACT CATTGGACTC CGATACCGTC CCGGACGGCG
```

FIG. 6G

```
      3010       3020       3030       3040       3050       3060
CCCGACGTTG GCTGCGAGCC CTGGGCCTTC ACCCGAACTT GGGGGGTGGG GTGGGGAAAA
GGGCTGCAAC CGACGCTCGG GACCCGGAAG TGGGCTTGAA CCCCCCACCC CACCCCTTTT

      3070       3080       3090       3100       3110       3120
GGAAGAAACG CGGGCGTATT GGCCCCAATG GGGTCTCGGT GGGGTATCGA CAGAGTGCCA
CCTTCTTTGC GCCCGCATAA CCGGGGTTAC CCCAGAGCCA CCCCATAGCT GTCTCACGGT

      3130       3140       3150       3160       3170       3180
GCCCTGGGAC CGAACCCCGC GTTTATGAAC AAACGACCCA ACACCGTGCG TTTTATTCTG
CGGGACCCTG GCTTGGGGCG CAAATACTTG TTTGCTGGGT TGTGGCACGC AAAATAAGAC

      3190       3200       3210       3220       3230       3240
TCTTTTTATT GCCGTCATAG CGCGGGTTCC TTCCGGTATT GTCTCCTTCC GTGTTTCAGT
AGAAAAATAA CGGCAGTATC GCGCCCAAGG AAGGCCATAA CAGAGGAAGG CACAAAGTCA

      3250       3260       3270       3280       3290       3300
TAGCCTCCCC CTAGGGTGGG CGAAGAACTC CAGCATGAGA TCCCCGCGCT GGAGGATCAT
ATCGGAGGGG GATCCCACCC GCTTCTTGAG GTCGTACTCT AGGGGCGCGA CCTCCTAGTA

      3310       3320       3330       3340       3350       3360
CCAGCCGGCG TCCCGGAAAA CGATTCCGAA GCCCAACCTT TCATAGAAGG CGGCGGTGGA
GGTCGGCCGC AGGGCCTTTT GCTAAGGCTT CGGGTTGGAA AGTATCTTCC GCCGCCACCT

      3370       3380       3390       3400       3410       3420
ATCGAAATCT CGTGATGGCA GGTTGGGCGT CGCTTGGTCG GTCATTTCGA ACCCCAGAGT
TAGCTTTAGA GCACTACCGT CCAACCCGCA GCGAACCAGC CAGTAAAGCT TGGGGTCTCA

      3430       3440       3450       3460       3470       3480
CCCGCTCAGA AGAACTCGTC AAGAAGGCGA TAGAAGGCGA TGCGCTGCGA ATCGGGAGCG
```

FIG. 6H

```
GGGCGAGTCT TCTTGAGCAG TTCTTCCGCT ATCTTCCGCT ACGCGACGCT TAGCCCTCGC

      3490        3500        3510        3520        3530        3540
GCGATACCGT AAAGCACGAG GAAGCGGTCA GCCCATTCGC CGCCAAGCTC TTCAGCAATA
CGCTATGGCA TTTCGTGCTC CTTCGCCAGT CGGGTAAGCG GCGGTTCGAG AAGTCGTTAT

      3550        3560        3570        3580        3590        3600
TCACGGGTAG CCAACGCTAT GTCCTGATAG CGGTCCCGCA CACCCAGCCG GCCACAGTCG
AGTGCCCATC GGTTGCGATA CAGGACTATC GCCAGGCCGT GTGGGTCGGC CGGTGTCAGC

      3610        3620        3630        3640        3650        3660
ATGAATCCAG AAAAGCGGCC ATTTTCCACC ATGATATTCG GCAAGCAGGC ATCGCCATGG
TACTTAGGTC TTTTCGCCGG TAAAAGGTGG TACTATAAGC CGTTCGTCCG TAGCGGTACC

      3670        3680        3690        3700        3710        3720
GTCACGACGA GATCCTCGCC GTCGGGCATG CTCGACCTTG A GCCTGGCGAA CAGTTCGGCT
CAGTGCTGCT CTAGGAGCGG CAGCCCGTAC GAGCGGAACT CGGACCGCTT GTCAAGCCGA

      3730        3740        3750        3760        3770        3780
GGCGCGAGCC CCTGATGCTC TTGATCATCC TGATCGACAA GACCGGCTTC CATCCGAGTA
CCGCGCTCGG GGACTACGAG AACTAGTAGG ACTAGCTGTT CTGGCCGAAG GTAGGCTCAT

      3790        3800        3810        3820        3830        3840
CGTGCTCGCT CGATGCGATG TTTCGCTTGG TGGTCGAATG GGCAGGTAGC CGGATCAAGC
GCACGAGCGA GCTACGCTAC AAAGCGAACC ACCAGCTTAC CCGTCCATCG GCCTAGTTCG

      3850        3860        3870        3880        3890        3900
GTATGCAGCC GCCGCATTGC ATCAGCCATG ATGGATACTT TCTCGGCAGG AGCAAGGTGA
CATACGTCGG CGGCGTAACG TAGTCGGTAC TACCTATGAA AGAGCCGTCC TCGTTCCACT
```

FIG. 6I

```
3910        3920        3930        3940        3950        3960
GATGACAGGA GATCCTGCCC CGGCACTTCG CCCAATAGCA GCCAGTCCCT TCCCGCTTCA
CTACTGTCCT CTAGGACGGG GCCGTGAAGC GGGTTATCGT CGGTCAGGGA AGGGCGAAGT

3970        3980        3990        4000        4010        4020
GTGACAACGT CGAGCACAGC TGCGCAAGGA ACGCCCGTCG TGGCCAGCCA CGATAGCCGC
CACTGTTGCA GCTCGTGTCG ACGCGTTCCT TGCGGGCAGC ACCGGTCGGT GCTATCGGCG

4030        4040        4050        4060        4070        4080
GCTGCCTCGT CTTGCAGTTC ATTCAGGGCA CCGGACAGGT CGGTCTTGAC AAAAAGAACC
CGACGGAGCA GAACGTCAAG TAAGTCCCGT GGCCTGTCCA GCCAGAACTG TTTTTCTTGG

4090        4100        4110        4120        4130        4140
GGGCGCCCCT GCGCTGACAG CCGGAACACG GCGGCATCAG AGCAGCCGAT TGTCTGTGT
CCCGCGGGGA CGCGACTGTC GGCCTTGTGC CGCCGTAGTC TCGTCGGCTA ACAGACAACA

4150        4160        4170        4180        4190        4200
GCCCAGTCAT AGCCGAATAG CCTCTCCACC CAAGCGGCCG GAGAACCTGC GTGCAATCCA
CGGGTCAGTA TCGGCTTATC GGAGAGGTGG GTTCGCCGGC CTCTTGGACG CACGTTAGGT

4210        4220        4230        4240        4250        4260
TCTTGTTCAA TCATGCGAAA CGATCCTCAT CCTGTCTCTT GATCGATCTT TGCAAAAGCC
AGAACAAGTT AGTACGCTTT GCTAGGAGTA GGACAGAGAA CTAGCTAGAA ACGTTTTCGG

4270        4280        4290        4300        4310        4320
TAGGCCTCCA AAAAAGCCTC CTCACTACTT CTGGAATAGC TCAGAGGCCG AGGCGGCCTC
ATCCGGAGGT TTTTTCGGAG GAGTGATGAA GACCTTATCG AGTCTCCGGC TCCGCCGGAG

4330        4340        4350        4360        4370        4380
GGCCTCTGCA TAAATAAAAA AAATTAGTCA GCCATGGGGC GGAGAATGGG CGGAACTGGG
CCGGAGACGT ATTTATTTTT TTTAATCAGT CGGTACCCCG CCTCTTACCC GCCTTGACCC

4390        4400        4410        4420        4430        4440
```

FIG. 6J

```
CGGAGTTAGG GGCGGGATGG GCGGAGTTAG GGGCGGGACT ATGGTTGCTG ACTAATTGAG
GCCTCAATCC CCGCCCTACC CGCCTCAATC CCCGCCCCTGA TACCAACGAC TGATTAACTC

      4450       4460       4470       4480       4490       4500
ATGCATGCTT TGCATACTTC TGCCTGCTGG GGAGCCTGGG GACTTTCCAC ACCTGGTTGC
TACGTACGAA ACGTATGAAG ACGGACGACC CCTCGGACCC CTGAAAGGTG TGGACCAACG

      4510       4520       4530       4540       4550       4560
TGACTAATTG AGATGCATGC TTTGCATACT TCTGCCTGCT GGGGAGCCTG GGGACTTTCC
ACTGATTAAC TCTACGTACG AAACGTATGA AGACGGACGA CCCCTCGGAC CCCTGAAAGG

      4570       4580       4590       4600       4610       4620
ACACCCTAAC TGACACACAT TCCACAGCTG GTTCTTTCCG CCTCAGGACT CTTCCTTTTT
TGTGGGATTG ACTGTGTGTA AGGTGTCGAC CAAGAAAGGC GGAGTCCTGA GAAGGAAAAA

      4630       4640       4650       4660       4670       4680
CAATAAATCA ATCTAAAGTA TATATGAGTA AACTTGGTCT GACAGTTACC AATGCTTAAT
GTTATTTAGT TAGATTTCAT ATATACTCAT TTGAACCAGA CTGTCAATGG TTACGAATTA

      4690       4700       4710       4720       4730       4740
CAGTGAGGCA CCTATCTCAG CGATCTGTCT ATTTCGTTCA TCCATAGTTG CCTGACTCCC
GTCACTCCGT GGATAGAGTC GCTAGACAGA TAAAGCAAGT AGGTATCAAC GGACTGAGGG

      4750       4760       4770       4780       4790       4800
CGTCGTGTAG ATAACTACGA TACGGGAGGG CTTACCATCT GGCCCCAGTG CTGCAATGAT
GCAGCACATC TATTGATGCT ATGCCCTCCC GAATGGTAGA CCGGGGTCAC GACGTTACTA

      4810       4820       4830       4840       4850       4860
ACCGCGAGAC CCACGCTCAC CGGCTCCAGA TTTATCAGCA ATAAACCAGC CAGCCGGAAG
TGGCGCTCTG GGTGCGAGTG GCCGAGGTCT AAATAGTCGT TATTTGGTCG GTCGGCCTTC
```

FIG. 6K

```
4870       4880       4890       4900       4910       4920
GGCCGAGCGC AGAAGTGGTC CTGCAACTTT ATCCGCCTCC ATCCAGTCTA TTAATTGTTG
CCGGCTCGCG TCTTCACCAG GACGTTGAAA TAGGCGGAGG TAGGTCAGAT AATTAACAAC

4930       4940       4950       4960       4970       4980
CCGGGAAGCT AGAGTAAGTA GTTCGCCAGT TAATAGTTTG CGCAACGTTG TTGCCATTGC
GGCCCTTCGA TCTCATTCAT CAAGCGGTCA ATTATCAAAC GCGTTGCAAC AACGGTAACG

4990       5000       5010       5020       5030       5040
TACAGGCATC GTGGTGTCAC GCTCGTCGTT TGGTATGGCT TCATTCAGCT CCGGTTCCCA
ATGTCCGTAG CACCACAGTG CGAGCAGCAA ACCATACCGA AGTAAGTCGA GGCCAAGGGT

5050       5060       5070       5080       5090       5100
ACGATCAAGG CGAGTTACAT GATCCCCCAT GTTGTGCAAA AAAGCGGTTA GCTCCTTCGG
TGCTAGTTCC GCTCAATGTA CTAGGGGGTA CAACACGTTT TTTCGCCAAT CGAGGAAGCC

5110       5120       5130       5140       5150       5160
TCCTCCGATC GTTGTCAGAA GTAAGTTGGC CGCAGTGTTA TCACTCATGG TTATGGCAGC
AGGAGGCTAG CAACAGTCTT CATTCAACCG GCGTCACAAT AGTGAGTACC AATACCGTCG

5170       5180       5190       5200       5210       5220
ACTGCATAAT TCTCTTACTG TCATGCCATC CGTAAGATGC TTTTCTGTGA CTGGTGAGTA
TGACGTATTA AGAGAATGAC AGTACGGTAG GCATTCTACG AAAAGACACT GACCACTCAT

5230       5240       5250       5260       5270       5280
CTCAACCAAG TCATTCTGAG AATAGTGTAT GCGGCGACCG AGTTGCTCTT GCCCGGCGTC
GAGTTGGTTC AGTAAGACTC TTATCACATA CGCCGCTGGC TCAACGAGAA CGGGCCGCAG

5290       5300       5310       5320       5330       5340
AATACGGGAT AATACCGCGC CACATAGCAG AACTTTAAAA GTGCTCATCA TTGGAAAACG
TTATGCCCTA TTATGGCGCG GTGTATCGTC TTGAAATTTT CACGAGTAGT AACCTTTTGC
```

FIG. 6L

```
                5350        5360        5370        5380        5390        5400
TTCTTCGGGG CGAAAACTCT CAAGGATCTT ACCGCTGTTG AGATCCAGTT CGATGTAACC
AAGAAGCCCC GCTTTTGAGA GTTCCTAGAA TGGCGACAAC TCTAGGTCAA GCTACATTGG

                5410        5420        5430        5440        5450        5460
CACTCGTGCA CCCAACTGAT CTTCAGCATC TTTTACTTTC ACCAGCGTTT CTGGGTGAGC
GTGAGCACGT GGGTTGACTA GAAGTCGTAG AAAATGAAAG TGGTCGCAAA GACCCACTCG

                5470        5480        5490        5500        5510        '5520
AAAAACAGGA AGGCAAAATG CCGCAAAAAA GGGAATAAGG GCGACACGGA AATGTTGAAT
TTTTTGTCCT TCCGTTTTAC GGCGTTTTTT CCCTTATTCC CGCTGTGCCT TTACAACTTA

                5530        5540        5550        5560        5570        5580
ACTCATACTC TTCCTTTTTC AATATTATTG AAGCATTTAT CAGGGTTATT GTCTCATGAG
TGAGTATGAG AAGGAAAAAG TTATAATAAC TTCGTAAATA GTCCCAATAA CAGAGTACTC

                5590        5600        5610        5620        5630        5640
CGGATACATA TTTGAATGTA TTTAGAAAAA TAAACAAATA GGGGTTCCGC GCACATTTCC
GCCTATGTAT AAACTTACAT AAATCTTTTT ATTTGTTTAT CCCCAAGGCG CGTGTAAAGG

                5650        5660        5670        5680        5690        5700
CCGAAAAGTG CCACCTGACG CGCCCTGTAG CGGCGCATTA AGCGCGGCGG GTGTGGTGGT
GGCTTTTCAC GGTGGACTGC GCGGGACATC GCCGCGTAAT TCGCGCCGCC CACACCACCA
```

FIG. 6M

```
                5710        5720        5730        5740        5750        5760
TACGCGCAGC GTGACCGCTA CACTTCCCAG CGCCCTAGCG CCCGCTCCTT TCGCTTTCTT
ATGCGCGTCG CACTGGCGAT GTGAACGGTC GCGGGATCGC GGGCGAGGAA AGCGAAAGAA

                5770        5780        5790        5800        5810        5820
CCCTTCCTTT CTCGCCACGT TCGCCGGCTT TCCCCGTCAA GCTCTAAATC GGGGGCTCCC
GGGAAGGAAA GAGCGGTGCA AGCGGCCGAA AGGGGCAGTT CGAGATTTAG CCCCCGAGGG

                5830        5840        5850        5860        5870        5880
TTTAGGGTTC CGATTTAGTG CTTTACGGCA CCTCGACCCC AAAAAACTTG ATTAGGGTGA
AAATCCCAAG GCTAAATCAC GAAATGCCGT GGAGCTGGGG TTTTTTGAAC TAATCCCACT

                5890        5900        5910        5920        5930        5940
TGGTTCACGT AGTGGGCCAT CGCCCTGATA GACGGTTTTT CGCCCTTTGA CGTTGGAGTC
ACCAAGTGCA TCACCCGGTA GCGGGACTAT CTGCCAAAAA GCGGGAAACT GCAACCTCAG

                5950        5960        5970        5980        5990        6000
CACGTTCTTT AATAGTGGAC TCTTGTTCCA AACTGGAACA ACACTCAACC CTATCTCGGT
GTGCAAGAAA TTATCACCTG AGAACAAGGT TTGACCTTGT TGTGAGTTGG GATAGAGCCA

                6010        6020        6030        6040        6050        6060
CTATTCTTTT GATTTATAAG GGATTTTGCC GATTTCGGCC TATTGGTTAA AAAATGAGCT
GATAAGAAAA CTAAATATTC CCTAAAACGG CTAAAGCCGG ATAACCAATT TTTTACTCGA

                6070        6080        6090        6100        6110        6120
GATTTAACAA AAATTTAACG CGAATTTTAA CAAAATATTA ACGCTTACAA TTTAC.....
CTAAATTGTT TTTAAATTGC GCTTAAAATT GTTTTATAAT TGCGAATGTT AAATG.....
```

FIG. 6N

CMV promotor: bases 1-596
Putative Transcriptional Start: bases 620-625
T7 promotor: bases 638-657
Multiple Cloning Site: bases 664-769
SP6 promotor: bases 774-791
BGH poly A: bases 796-1024
ColE1 origin: bases 1155-1738
TK poly A signal: bases 1923-2194
Kanamycin/Neomycin resistance: bases 2195-3191
SV40 promotor/origin: bases 3192-3549
Ampicillin Resistance: bases 3568-4599
F1 origin: bases 4600-5056

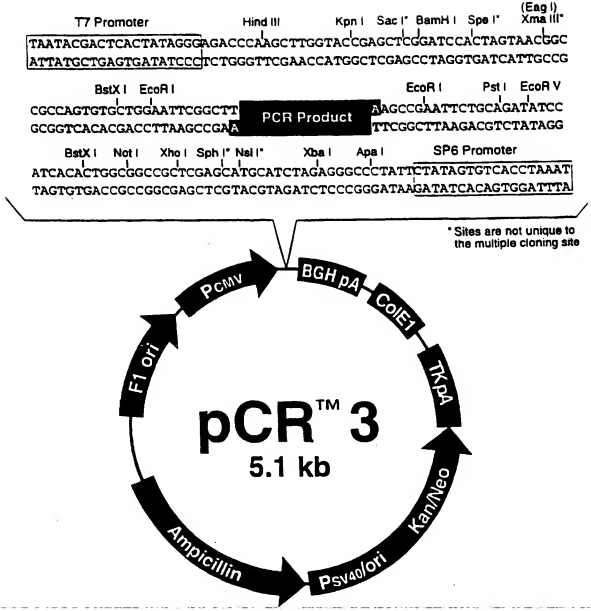


FIG. 7A

```
CMV (1-596), T7 (638-657), MCS (664-718), LacZ (728-3787*)
MCS (3791-3847) Kan (6235-5447) Amp (7547-6687)
*NOTE:  3' sequence of LacZ may not be exact)
        10        20        30        40        50        60
GCGCGCGTTG ACATTGATTA TTGACTAGTT ATTAATAGTA ATCAATTACG GGGTCATTAG

        70        80        90       100       110       120
TTCATAGCCC ATATATGGAG TTCCGCGTTA CATAACTTAC GGTAAATGGC CCGCCTGGCT

       130       140       150       160       170       180
GACCGCCCAA CGACCCCCGC CCATTGACGT CAATAATGAC GTATGTTCCC ATAGTAACGC

       190       200       210       220       230       240
CAATAGGGAC TTTCCATTGA CGTCAATGGG TGGACTATTT ACGGTAAACT GCCCACTTGG

       250       260       270       280       290       300
CAGTACATCA AGTGTATCAT ATGCCAAGTA CGCCCCCTAT TGACGTCAAT GACGGTAAAT

       310       320       330       340       350       360
GGCCCGCCTG GCATTATGCC CAGTACATGA CCTTATGGGA CTTTCCTACT TGGCAGTACA

       370       380       390       400       410       420
TCTACGTATT AGTCATCGCT ATTACCATGG TGATGCGGTT TTGGCAGTAC ATCAATGGGC
```

FIG. 7B

```
          430        440        450        460        470        480
GTGGATAGCG GTTTGACTCA CGGGGATTTC CAAGTCTCCA CCCCATTGAC GTCAATGGGA

          490        500        510        520        530        540
GTTTGTTTTG GCACCAAAAT CAACGGACTT TTCCAAAATG TCGTAACAAC TCCGCCCCAT

          550        560        570        580        590        600
TGACGCAAAT GGGCGGTAGG CGTGTACGGT GGGAGGTCTA TATAAGCAGA GCTCTCTGGC

          610        620        630        640        650        660
TAACTAGAGA ACCCACTGCT TACTGGCTTA TCGAAATTAA TACGACTCAC TATAGGGAGA

          670        680        690        700        710        720
CCCAAGCTTG GTACCGAGCT CGGATCCACT AGTAACGGCC GCCAGTGTGC TGGAATTCGG

          730        740        750        760        770        780
CTTATTCATG ATAGATCCCG TCGTTTTACA ACGTCGTGAC TGGGAAAACC CTGGCGTTAC

          790        800        810        820        830        840
CCAACTTAAT CGCCTTGCAG CACATCCCCC TTTCGCCAGC TGGCGTAATA GCGAAGAGGC

          850        860        870        880        890        900
CCGCACCGAT CGCCCTTCCC AACAGTTGCG CAGCCTGAAT GGCGAATGGC GCTTTGCCTG
```

FIG. 7C

```
      910       920       930       940       950       960
GTTTCCGGTA CCAGAAGCGG TGCCCGGAAAG CTGGCTGGAG TGCGATCTTC CTGAGGCCGA

      970       980       990      1000      1010      1020
TACTGTCGTC GTCCCCTCAA ACTGGCAGAT GCACGGTTAC GATGCGCCCA TCTACACCAA

     1030      1040      1050      1060      1070      1080
CGTAACCTAT CCCATTACGG TCAATCCGCC GTTTGTTCCC ACGGAGAATC CGACGGGTTG

     1090      1100      1110      1120      1130      1140
TTACTCGCTC ACATTTAATG TTGATGAAAG CTGGCTACAG GAAGGCCAGA CGCGAATTAT

     1150      1160      1170      1180      1190      1200
TTTTGATGGC GTTAACTCGG CGTTTCATCT GTGGTGCAAC GGGCGCTGGG TCGGTTACGG

     1210      1220      1230      1240      1250      1260
CCAGGACAGT CGTTTGCCGT CTGAATTTGA CCTGAGCGCA TTTTTACGCG CCGGAGAAAA

     1270      1280      1290      1300      1310      1320
CCGCCTCGCG GTGATGGTGC TGCGTTGGAG TGACGGCAGT TATCTGGAAG ATCAGGATAT

     1330      1340      1350      1360      1370      1380
GTGGCGGATG AGCGGCATTT TCCGTGACGT CTCGTTGCTG CATAAACCGA CTACACAAAT
```

FIG. 7D

```
1390                1400                1410                1420                1430                1440
CAGCGATTTC CATGTTGCCA CTCGCTTTAA TGATGATTTC AGCCGCGCTG TACTGGAGGC

1450                1460                1470                1480                1490                1500
TGAAGTTCAG ATGTGCGGCG AGTTGCGTGA CTACCTACGG GTAACAGTTT CTTTATGGCA

1510                1520                1530                1540                1550                1560
GGGTGAAACG CAGGTCGCCA GCGGCACCGC GCCTTTCGGC GGTGAAATTA TCGATGAGCG

1570                1580                1590                1600                1610                1620
TGGTGGTTAT GCCGATCGCG TCACACTACG TCTGAACGTC GAAAACCCGA AACTGTGGAG

1630                1640                1650                1660                1670                1680
CGCCGAAATC CCGAATCTCT ATCGTGCGGT GGTTGAACTG CACACCGCCG ACGGCACGCT

1690                1700                1710                1720                1730                1740
GATTGAAGCA GAAGCCTGCG ATGTCGGTTT CCGCGAGGTG CGGATTGAAA ATGGTCTGCT

1750                1760                1770                1780                1790                1800
GCTGCTGAAC GGCAAGCCGT TGCTGATTCG AGGCGTTAAC CGTCACGAGC ATCATCCTCT

1810                1820                1830                1840                1850                1860
GCATGGTCAG GTCATGGATG AGCAGACGAT GGTGCAGGAT ATCCTGCTGA TGAAGCAGAA
```

FIG. 7E

```
1870      1880      1890      1900      1910      1920
CAACTTTAAC GCCGTGCGCT GTTCGCATTA TCCGAACCAT CCGCTGTGGT ACACGCTGTG

1930      1940      1950      1960      1970      1980
CGACCGCTAC GGCCTGTATG TGGTGGATGA AGCCAATATT GAAACCCACG GCATGGTGCC

1990      2000      2010      2020      2030      2040
AATGAATCGT CTGACCGATG ATCCGCGCTG GCTACCGGCG ATGAGCGAAC GCGTAACGCG

2050      2060      2070      2080      2090      2100
AATGGTGCAG CGCGATCGTA ATCACCCGAG TGTGATCATC TGGTCGCTGG GGAATGAATC

2110      2120      2130      2140      2150      2160
AGGCCACGGC GCTAATCACG ACGCGCTGTA TCGCTGGATC AAATCTGTCG ATCCTTCCCG

2170      2180      2190      2200      2210      2220
CCCGGTGCAG TATGAAGGCG GCGGAGCCGA CACCACGGCC ACCGATATTA TTTGCCCGAT

2230      2240      2250      2260      2270      2280
GTACGCGCGC GTGGATGAAG ACCAGCCCTT CCCGGCTGTG CCGAAATGGT CCATCAAAAA

2290      2300      2310      2320      2330      2340
ATGGCTTTCG CTACCTGGAG AGACGCCCCC GCTGATCCTT TGCGAATACG CCCACGCGAT
```

FIG. 7F

```
      2350       2360       2370       2380       2390       2400
GGGTAACAGT CTTGGCGGTT TCGCTAAATA CTGGCAGGCG TTTCGTCAGT ATCCCCGTTT

      2410       2420       2430       2440       2450       2460
ACAGGGCGGC TTCGTCTGGG ACTGGGTGGA TCAGTCGCTG ATTAAATATG ATGAAAACGG

      2470       2480       2490       2500       2510       2520
CAACCCGTGG TCGGCTTACG GCGGTGATTT TGGCGATACG CCGAACGATC GCCAGTTCTG

      2530       2540       2550       2560       2570       2580
TATGAACGGT CTGGTCTTTG CCGACCGCAC GCCGCATCCA GCGCTGACGG AAGCAAAACA

      2590       2600       2610       2620       2630       2640
CCAGCAGCAG TTTTTCCAGT TCCGTTTATC CGGGCAAACC ATCGAAGTGA CCAGCGAATA

      2650       2660       2670       2680       2690       2700
CCTGTTCCGT CATAGCGATA ACGAGCTCCT GCACTGGATG GTGGCGCTGG ATGGTAAGCC

      2710       2720       2730       2740       2750       2760
GCTGGCAAGC GGTGAAGTGC CTCTGGATGT CGCTCCACAA GGTAAACAGT TGATTGAACT

      2770       2780       2790       2800       2810       2820
GCCTGAACTA CCGCAGCCGG AGAGCGCCGG GCAACTCTGG CTCACAGTAC GCGTAGTGCA
```

FIG. 7G

```
2830       2840       2850       2860       2870       2880
ACCGAACGCG ACCGCATGGT CAGAAGCCGG GCACATCAGC GCCTGGCAGC AGTGGCGTCT

2890       2900       2910       2920       2930       2940
GGCGGAAAAC CTCAGTGTGA CGCTCCCCGC CGCGTCCCAC GCCATCCCGC ATCTGACCAC

2950       2960       2970       2980       2990       3000
CAGCGAAATG GATTTTTGCA TCGAGCTGGG TAATAAGCGT TGGCAATTTA ACCGCCAGTC

3010       3020       3030       3040       3050       3060
AGGCTTTCTT TCACAGATGT GGATTGGCGA TAAAAAACAA CTGCTGACGC CGCTGCGCGA

3070       3080       3090       3100       3110       3120
TCAGTTCACC CGTGCACCGC TGGATAACGA CATTGGCGTA AGTGAAGCGA CCCGCATTGA

3130       3140       3150       3160       3170       3180
CCCTAACGCC TGGGTCGAAC GCTGGAAGGC GGCGGGCCAT TACCAGGCCG AAGCAGCGTT

3190       3200       3210       3220       3230       3240
GTTGCAGTGC ACGGCAGATA CACTTGCTGA TGCGGTGCTG ATTACGACCG CTCACGCGTG
```

FIG. 7H

```
3250      3260      3270      3280      3290      3300
GCAGCATCAG GGGAAAACCT TATTTATCAG CCGGAAAACC TACCGGATTG ATGGTAGTGG

3310      3320      3330      3340      3350      3360
TCAAATGGCG ATTACCGTTG ATGTTGAAGT GGCGACGCGAT ACACCGCATC CGGCGCGGAT

3370      3380      3390      3400      3410      3420
TGGCCTGAAC TGCCAGCTGG CGCAGGTAGC AGAGCGGGTA AACTGGCTCG GATTAGGGCC

3430      3440      3450      3460      3470      3480
GCAAGAAAAC TATCCCGACC GCCTTACTGC CGCCTGTTTT GACCGCTGGG ATCTGCCATT

3490      3500      3510      3520      3530      3540
GTCAGACATG TATACCCCGT ACGTCTTCCC GAGCGAAAAC GGTCTGCGCT GCGGGACGCG

3550      3560      3570      3580      3590      3600
CGAATTGAAT TATGGCCCAC ACCAGTGGCG CGGCGACTTC CAGTTCAACA TCAGCCGCTA

3610      3620      3630      3640      3650      3660
CAGTCAACAG CAACTGATGG AAACCAGCCA TCGCCATCTG CTGCACGCGG AAGAAGGCAC

3670      3680      3690      3700      3710      3720
ATGGCTGAAT ATCGACGGTT TCCATATGGG GATTGGTGGC GACGACTCCT GGAGCCCGTC

3730      3740      3750      3760      3770      3780
```

FIG. 71

```
AGTATCGGCG GAATTCCAGC TGAGCGCCGG TCGCTACCAT TACCAGTTGG TCTGGTGTCA

      3790       3800       3810       3820       3830       3840
AAAATAAGCC GAATTCTGCA GATATCCATC ACACTGGCGG CCGCTCGAGC ATGCATCTAG

      3850       3860       3870       3880       3890       3900
AGGGCCCTAT TCTATAGTGT CACCTAAATG CTAGAGCTCG CTGATCAGCC TCGACTGTGC

      3910       3920       3930       3940       3950       3960
CTTCTAGTTG CCAGCCATCT GTTGTTTGCC CCTCCCCCGT GCCTTCCTTG ACCCTGGAAG

      3970       3980       3990       4000       4010       4020
GTGCCACTCC CACTGTCCTT TCCTAATAAA ATGAGGAAAT TGCATCGCAT TGTCTGAGTA

      4030       4040       4050       4060       4070       4080
GGTGTCATTC TATTCTGGGG GGTGGGGTGG GGCAGGACAG CAAGGGGGAG GATTGGGAAG

      4090       4100       4110       4120       4130       4140
ACAATAGCAG GCATGCTGGG GATGCGGTGG GCTCTATGGC TTCTGAGGCG GAAAGAACCA

      4150       4160       4170       4180       4190       4200
GTGGCGGTAA TACGGTTATC CACAGAATCA GGGGATAACG CAGGAAAGAA CATGTGAGCA
```

FIG. 7J

```
4210                4220                4230                4240                4250                4260
AAAGGCCAGC AAAAGGCCAG GAACCGTAAA AAGGCCGCGT TGCTGGCGTT TTTCCATAGG

4270                4280                4290                4300                4310                4320
CTCCGCCCCC CTGACGAGCA TCACAAAAAT CGACGCTCAA GTCAGAGGTG GCGAAACCCG

4330                4340                4350                4360                4370                4380
ACAGGACTAT AAAGATACCA GGCGTTTCCC CCTGGAAGCT CCCTCGTGCG CTCTCCTGTT

4390                4400                4410                4420                4430                4440
CCGACCCTGC CGCTTACCGG ATACCTGTCC GCCTTTCTCC CTTCGGGAAG CGTGGCGCTT

4450                4460                4470                4480                4490                4500
TCTCATAGCT CACGCTGTAG GTATCTCAGT TCGGTGTAGG TCGTTCGCTC CAAGCTGGGC

4510                4520                4530                4540                4550                4560
TGTGTGCACG AACCCCCCGT TCAGCCCGAC CGCTGCGCCT TATCCGGTAA CTATCGTCTT

4570                4580                4590                4600                4610                4620
GAGTCCAACC CGGTAAGACA CGACTTATCG CCACTGGCAG CAGCCACTGG TAACAGGATT

4630                4640                4650                4660                4670                4680
AGCAGAGCGA GGTATGTAGG CGGTGCTACA GAGTTCTTGA AGTGGTGGCC TAACTACGGC
```

FIG. 7K

```
4690       4700       4710       4720       4730       4740
TACACTAGAA GGACAGTAAT TGGTATCTGC GCTCTGCTGA AGCCAGTTAC CTTCGGAAAA

4750       4760       4770       4780       4790       4800
AGAGTTGGTA GCTCTTGATC CGGCAAACAA ACCACCGCTG GTAGCGGTGG TTTTTTTGTT

4810       4820       4830       4840       4850       4860
TGCAAGCAGC AGATTACGCG CAGAAAAAAA GGATCTCAAG AAGATCCTTT GATCTTTTCT

4870       4880       4890       4900       4910       4920
ACGGGGTCTG ACGCTCAGTG GAACGAAAAC TCACGTTAAG GGATTTTGGT CATGAGATTA

4930       4940       4950       4960       4970       4980
TCAAAAGGA TCTTCACCTA GATCCTTTTA AATTAAAAAT GAAGTTTTAA ATCAATCTAA

4990       5000       5010       5020       5030       5040
AGTATATATG AGTAACCTGA GGCTATGGCA GGGCCTGCCG CCCCGACGTT GGCTGCGAGC

5050       5060       5070       5080       5090       5100
CCTGGGCCTT CACCCGAACT TGGGGGGTGG GGTGGGGAAA AGGAAGAAAC GCGGGCGTAT

5110       5120       5130       5140       5150       5160
TGGCCCCAAT GGGGTCTCGG TGGGGTATCG ACAGAGTGCC AGCCCTGGGA CCGAACCCCG
```

FIG. 7L

```
5170       5180       5190       5200       5210       5220
CGTTTATGAA CAAACGACCC AACACCGTGC GTTTTATTCT GTCTTTTTAT TGCCGTCATA

5230       5240       5250       5260       5270       5280
GCGCGGGTTC CTTCCGGTAT TGTCTCCTTC CGTGTTTCAG TTAGCCTCCC CCTAGGGTGG

5290       5300       5310       5320       5330       5340
GCGAAGAACT CCAGCATGAG ATCCCCGCGC TGGAGGATCA TCCAGCCGGC GTCCCGGAAA

5350       5360       5370       5380       5390       5400
ACGATTCCGA AGCCCAACCT TTCATAGAAG GCGGCGGTGG AATCGAAATC TCGTGATGGC

5410       5420       5430       5440       5450       5460
AGGTTGGGCG TCGCTTGGTC GGTCATTTCG AACCCCAGAG TCCCGCTCAG AAGAACTCGT

5470       5480       5490       5500       5510       5520
CAAGAAGGCG ATAGAAGGCG ATGCGCTGCG AATCGGGAGC GGCGATACCG TAAAGCACGA

5530       5540       5550       5560       5570       5580
GGAAGCGGTC AGCCCATTCG CCGCCAAGCT CTTCAGCAAT ATCACGGGTA GCCAACGCTA

5590       5600       5610       5620       5630       5640
TGTCCTGATA GCGGTCCGCC ACACCCAGCC GGCCACAGTC GATGAATCCA GAAAAGCGGC
```

FIG. 7M

```
5650      5660      5670      5680      5690      5700
CATTTTCCAC CATGATATTC GGCAAGCAGG CATCGCCATG GGTCACGACG AGATCCTCGC

5710      5720      5730      5740      5750      5760
CGTCGGGCAT GCTCGCCTTG AGCCTGGCGA ACAGTTCGGC TGGCGCGAGC CCCTGATGCT

5770      5780      5790      5800      5810      5820
CTTGATCATC CTGATCGACA AGACCGGCTT CCATCCGAGT ACGTGCTCGC TCGATGCGAT

5830      5840      5850      5860      5870      5880
GTTTCGCTTG GTGGTCGAAT GGGCAGGTAG CCGGATCAAG CGTATGCAGC CGCCGCATTG

5890      5900      5910      5920      5930      5940
CATCAGCCAT GATGGATACT TTCTCGGCAG GAGCAAGGTG AGATGACAGG AGATCCTGCC

5950      5960      5970      5980      5990      6000
CCGGCACTTC GCCCAATAGC AGCCAGTCCC TTCCCGCTTC AGTGACAACG TCGAGCACAG

6010      6020      6030      6040      6050      6060
CTGCGCAAGG AACGCCCGTC GTGGCCAGCC ACGATAGCCG CGCTGCCTCG TCTTGCAGTT

6070      6080      6090      6100      6110      6120
CATTCAGGGC ACCGGACAGG TCGGTCTTGA CAAAAAGAAC CGGGCGCCCC TGCGCTGACA
```

FIG. 7N

```
6130                6140                6150                6160                6170                6180
GCCGGAACAC GGCGGCATCA GAGCAGCCGA TTGTCTGTTG TGCCCAGTCA TAGCCGAATA

6190                6200                6210                6220                6230                6240
GCCTCTCCAC CCAAGCGGCC GGAGAACCTG CGTGCAATCC ATCTTGTTCA ATCATGCGAA

6250                6260                6270                6280                6290                6300
ACGATCCTCA TCCTGTCTCT TGATCGATCT TTGCAAAAGC CTAGGCCTCC AAAAAAGCCT

6310                6320                6330                6340                6350                6360
CCTCACTACT TCTGGAATAG CTCAGAGGCC GAGGCGGCCT CGGCCTCTGC ATAAATAAAA

6370                6380                6390                6400                6410                6420
AAAATTAGTC AGCCATGGGG CGGAGAATGG GCGGAACTGG GCGGAGTTAG GGGCGGGATG

6430                6440                6450                6460                6470                6480
GGCGGAGTTA GGGGCGGGAC TATGGTTGCT GACTAATTGA GATGCATGCT TTGCATACTT

6490                6500                6510                6520                6530                6540
CTGCCTGCTG GGGAGCCTGG GGACTTTCCA CACCTGGTTG CTGACTAATT GAGATGCATG

6550                6560                6570                6580                6590                6600
CTTTGCATAC TTCTGCCTGC TGGGGAGCCT GGGGACTTTC CACACCCTAA CTGACACACA
```

FIG. 7O

```
      6610       6620       6630       6640       6650       6660
TTCCACAGCT GGTTCTTTCC GCCTCAGGAC TCTTCCTTTT TCAATAAATC AATCTAAAGT

      6670       6680       6690       6700       6710       6720
ATATATGAGT AAACTTGGTC TGACAGTTAC CAATGCTTAA TCAGTGAGGC ACCTATCTCA

      6730       6740       6750       6760       6770       6780
GCGATCTGTC TATTTCGTTC ATCCATAGTT GCCTGACTCC CCGTCGTGTA GATAACTACG

      6790       6800       6810       6820       6830       6840
ATACGGGAGG GCTTACCATC TGGCCCCAGT GCTGCAATGA TACCGCGAGA CCCACGCTCA

      6850       6860       6870       6880       6890       6900
CCGGCTCCAG ATTTATCAGC AATAAACCAG CCAGCCGGAA GGGCCGAGCG CAGAAGTGGT

      6910       6920       6930       6940       6950       6960
CCTGCAACTT TATCCGCCTC CATCCAGTCT ATTAATTGTT GCCGGGAAGC TAGAGTAAGT

      6970       6980       6990       7000       7010       7020
AGTTCGCCAG TTAATAGTTT GCGCAACGTT GTTGCCATTG CTACAGGCAT CGTGGTGTCA
```

FIG. 7P

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7030        7040        7050        7060        7070        7080
CGCTCGTCGT TTGGTATGGC TTCATTCAGC TCCGGTTCCC AACGATCAAG GCGAGTTACA

7090        7100        7110        7120        7130        7140
TGATCCCCCA TGTTGTGCAA AAAAGCGGTT AGCTCCTTCG GTCCTCCGAT CGTTGTCAGA

7150        7160        7170        7180        7190        7200
AGTAAGTTGG CCGCAGTGTT ATCACTCATG GTTATGGCAG CACTGCATAA TTCTCTTACT

7210        7220        7230        7240        7250        7260
GTCATGCCAT CCGTAAGATG CTTTTCTGTG ACTGGTGAGT ACTCAACCAA GTCATTCTGA

7270        7280        7290        7300        7310        7320
GAATAGTGTA TGCGCGCACC GAGTTGCTCT TGCCCGGCGT CAATACGGGA TAATACCGCG

7330        7340        7350        7360        7370        7380
CCACATAGCA GAACTTTAAA AGTGCTCATC ATTGGAAAAC GTTCTTCGGG GCGAAAACTC

7390        7400        7410        7420        7430        7440
TCAAGGATCT TACCGCTGTT GAGATCCAGT TCGATGTAAC CCACTCGTGC ACCCAACTGA

7450        7460        7470        7480        7490        7500
TCTTCAGCAT CTTTTACTTT CACCAGCGTT TCTGGGTGAG CAAAAACAGG AAGGCAAAAT

7510        7520        7530        7540        7550        7560
```

FIG. 7Q

```
GCCGCAAAAA AGGGAATAAG GGCGACACGG AAATGTTGAA TACTCATACT CTTCCTTTTT

      7570       7580       7590       7600       7610       7620
CAATATTATT GAAGCATTTA TCAGGGTTAT TGTCTCATGA GCGGATACAT ATTTGAATGT

      7630       7640       7650       7660       7670       7680
ATTTAGAAAA ATAAACAAAT AGGGGTTCCG CGCACATTTC CCCGAAAAGT GCCACCTGAC

      7690       7700       7710       7720       7730       7740
GCGCCCTGTA GCGGCGCATT AAGCGCGGCG GGTGTGGTGG TTACGCGCAG CGTGACCGCT

      7750       7760       7770       7780       7790       7800
ACACTTGCCA GCGCCCTAGC GCCCGCTCCT TTCGCTTTCT TCCCTTCCTT TCTCGCCACG
```

FIG. 7R

```
      7810       7820       7830       7840       7850       7860
TTCGCCCGGCT TTCCCCCGTCA AGCTCTAAAT CGGGGGCTCC CTTTAGGGTT CCGATTTAGT

      7870       7880       7890       7900       7910       7920
GCTTTACGGC ACCTCGACCC CAAAAAACTT GATTAGGGTG ATGGTTCACG TAGTGGGCCA

      7930       7940       7950       7960       7970       7980
TCGCCCTGAT AGACGGTTTT TCGCCCTTTG ACGTTGGAGT CCACGTTCTT TAATAGTGGA

      7990       8000       8010       8020       8030       8040
CTCTTGTTCC AAACTGGAAC AACACTCAAC CCTATCTCGG TCTATTCTTT TGATTTATAA

      8050       8060       8070       8080       8090       8100
GGGATTTTGC CGATTTCGGC CTATTGGTTA AAAAATGAGC TGATTTAACA AAAATTTAAC

      8110       8120       8130       8140       8150       8160
GCGAATTTTA ACAAAATATT AACGCTTACA ATTTAC.... .......... ..........
```

FIG. 7S

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/15819

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	CHESNUT, J.D. et al. Selective isolation of transiently transfected cells from a mammalian cell population with vectors expressing a membrane anchored single-chain antibody. J. Immunol. Meth. 14 June 1996, Vol. 193, pages 17-27, see entire document.	1-45
Y	HOOGENBOOM, H.R. et al. Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. Nucleic Acids Res. 1991, Vol. 19, No. 15, pages 4133-4137, see entire document.	1-45

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
19 DECEMBER 1996	17 JAN 1997

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	CHRISTOPHER EISENSCHENK
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US96/15819

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WILLIAMSON, R.A. et al. Human monoclonal antibodies against a plethora of viral pathogens from single combinatorial libraries. Proc. Natl. Acad. Sci. USA. 1993, Vol. 90, pages 4141-4145, see entire document.	1-45

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/15819

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07H 21/04; C12N 15/63, 15/85

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/240.1, 252.3, 320.1, 961

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

435/240.1, 252.3, 320.1, 961

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSYS, LIFESCI, EMBASE, WPI, MEDLINE